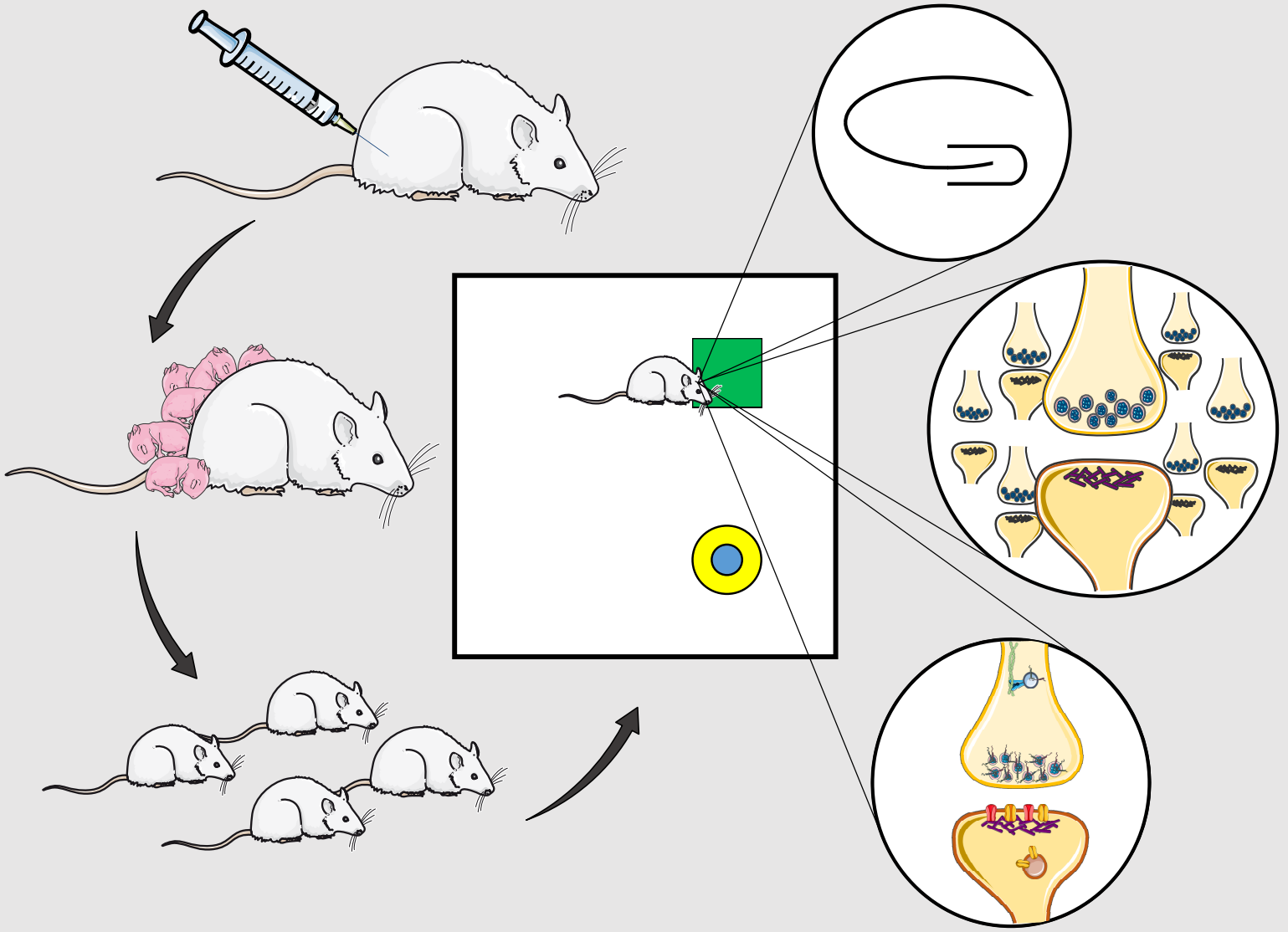

Fábio de Jesus Ribeiro de Sousa

IMPACT OF MATERNAL DIABETES ON OFFSPRING MEMORY

Dissertação de Mestrado em Biologia Celular e Molecular orientada pela Doutora Filipa Baptista e co-orientada pelo Doutor Carlos Duarte e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Setembro 2017

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UNIVERSIDADE DE COIMBRA

On the front page:

Schematic illustration of the main aims of the present thesis: the evaluation of the impact of maternal diabetes on offspring memory and correlation with hippocampal cellular and molecular alterations.

Impact of Maternal Diabetes on Offspring Memory

Fábio de Jesus Ribeiro de Sousa

Dissertation presented to the Faculty of Science and Technology of the University of Coimbra. The work was performed in the Retinal Dysfunction and Neuroinflammation Lab of the Institute for Biomedical Imaging and Life Science (IBILI), Faculty of Medicine, University of Coimbra, under the supervision of Doctor Filipa Baptista and co-supervision of Doctor Carlos Duarte.

University of Coimbra
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“Everything is theoretically impossible, until it is done”

Robert A. Heinlein

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Abbreviations List

A

AMPA	–	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	–	Alkaline phosphatase
APS	–	Ammonium persulfate

B

BCA	–	Bicinchoninic acid
BSA	–	Bovine serum albumin

C

CA	–	Cornu ammonis
CAPS	–	N-cyclohexyl-3-aminopropanesulfonic acid
CNS	–	Central nervous system
CTRL	–	Control

D

DG	–	Dentate gyrus
DOC	–	Sodium deoxycholate
DTT	–	Dithiothreitol

E

ECF	–	Enhanced chemifluorescence system
EDTA	–	Ethylenediaminetetracetic acid disodium salt dihydrate
EGTA	–	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
EPM	–	Elevated plus maze

G

GAPDH	–	Glyceraldehyde 3-phosphate dehydrogenase
GD	–	Gestation day
GW	–	Gestation week

H

HEPES	–	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	–	Horseradish peroxidase

I

IGF-1	–	Insulin-like growth factor-I

K

KHR	–	Krebs-Henseleit Ring

L

LDP	–	Long-term depression
LTP	–	Long-term potentiation

N

NMDA	–	N-methyl-D-aspartate
NOR	–	Novel object recognition

O

OPF	–	Open field

P

PBS	–	Phosphate buffered saline
PFA	–	Paraformaldehyde
PND	–	Postnatal day
PSD	–	Postsynaptic density
PVDF	–	Polyvinylidene difluoride

Q

Q/R	–	Glutamate to arginine

R

RIPA	–	Radioimmunoprecipitation assay
RT	–	Room temperature

S

SDS-PAGE	–	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	–	Standard error of the mean
STZ	–	Streptozotocin

T

TBS	–	Tris-buffered saline
TEMED	–	Tetramethylethylenediamine
Trizma-HCl	–	Tris(hydroxymethyl)aminomethane hydrochloride

V

VGluT	–	Vesicular glutamate transporters

Resumo

A diabetes durante a gravidez está associada a um aumento do risco de distúrbios do neurodesenvolvimento na descendência, nomeadamente défices psicomotores, sensoriais e cognitivos. Estudos experimentais indicam que diabetes materna induz modificações estruturais e funcionais no hipocampo da descendência, uma região cerebral associada aos processos de aprendizagem e memória. No entanto, os mecanismos exatos pelos quais o ambiente uterino de uma mãe diabética afeta o hipocampo da descendência permanecem por definir.

O objetivo principal da presente tese foi avaliar o impacto da diabetes materna na memória da descendência, avaliando primeiro o impacto no desenvolvimento durante o início da infância e depois analisando alterações celulares e moleculares subjacentes a alterações de memória. Além disso, as evidências sugerem que a diabetes materna afeta o cérebro da descendência de uma maneira dependente do género. No entanto, poucos estudos foram realizados simultaneamente em machos e fêmeas, a fim de estabelecer um efeito claro. Por isso, pretendemos também esclarecer o possível efeito diferencial da diabetes materna na descendência com base no género.

Para avaliar a influência do ambiente intrauterino da diabetes durante a gravidez no desenvolvimento durante as primeiras semanas de vida, foi realizada uma bateria de testes para avaliar reflexos, força e locomoção durante esse período, uma vez que alterações no desempenho nesses testes podem ser sugestivas de défices do desenvolvimento do sistema sensório-motor. A diabetes materna induziu um atraso no desenvolvimento de reflexos, força e comprometeu a locomoção durante as duas primeiras semanas pós-natais dos descendentes de ambos os sexos com tendência para um efeito mais evidente nos machos. O alcance de marcos físicos de desenvolvimento por parte dos descendentes foi avaliado, revelando também, um atraso no desenvolvimento. Adicionalmente, este estudo longitudinal demostrou que os descendentes de ambos os sexos de mães diabéticas apresentaram défices em termos de ganho de peso. Uma vez que o objetivo principal desta tese foi avaliar o impacto da diabetes materna na memória da descendência no final da infância e, tendo em consideração que a ansiedade pode afetar a cognição, avaliou-se o comportamento ansioso utilizando o teste de campo aberto (OPF) e o teste do labirinto em cruz elevado (EPM). A atividade locomotora também foi avaliada no OPF, uma vez que deficiências locomotoras podem influenciar o desempenho da descendência em testes de memória. Não foram observadas alterações na locomoção nos machos e fêmeas descendentes de mães diabéticas. Em relação ao comportamento ansioso, observou-se que a diabetes materna teve um impacto diferencial nos descendentes consoante o género.

As fêmeas demonstraram uma tendência a explorar menos a área central da arena do OPF e entraram menos vezes nos braços abertos do EPM, sugerindo um comportamento ansioso, enquanto nos machos não foram detetadas alterações. Para avaliar o impacto de diabetes materna na memória da descendência, realizou-se o teste de reconhecimento de objetos (NOR). Apesar de não ter sido possível realizar uma análise diferencial consoante o género, os resultados indicam que diabetes maternal afeta negativamente a memória da descendência.

Para investigar os mecanismos celulares e moleculares que podem estar subjacentes aos défices de memória dos descendentes, começou-se por avaliar a organização estrutural das sub-regiões do hipocampo através da coloração de violeta de cresilo. Não foram detetadas alterações na espessura das camadas nucleares das sub-regiões do hipocampo, sugerindo que a diabetes materna não induziu perda neuronal. Adicionalmente, avaliaram-se os níveis de proteínas envolvidas na sinaptogénese tais como a PSD-95, sinapsina-1, sinaptofisina e KIF1A. A diabetes materna não induziu alterações nos níveis de proteínas sinápticas incluindo nos níveis de VGluT-1, um marcador de sinapses glutamatérgicas. Os níveis proteicos de subunidades do recetores AMPA foram também avaliados uma vez que estes recetores são importantes para plasticidade sináptica. Observou-se uma tendência para a diminuição dos níveis proteicos totais de GluA1 no hipocampo de fêmeas, filhas de mães diabéticas e uma tendência para o aumento de GluA2 em sinaptossomas de hipocampo de machos. Estas alterações poderão afetar funcionalmente a neurotransmissão a plasticidade sináptica, e consequentemente os processos de memória.

Este trabalho realça a importância de compreender plenamente os efeitos deletérios da diabetes materna durante o desenvolvimento do cérebro, bem como o seu impacto a longo prazo em circuitos neuronais e no comportamento. Além disso, este estudo também demonstra a importância de estudar as diferenças de género em resposta a insultos durante o neurodesenvolvimento..

Uma melhor compreensão dessas questões permitirá delinear estratégias terapêuticas futuras de modo a prevenir e tratar a disfunção neuronal subjacente aos défices cognitivos e de memória da descendência das mães diabéticas.

Palavras-chave: Diabetes materna, Neurodesenvolvimento, Memória, Sinaptogénese, Diferenças de género

Abstract

Diabetes during pregnancy is associated with increased risk of neurodevelopmental disorders in the offspring, namely psychomotor, sensorial and cognitive impairments. Experimental studies indicate that diabetes during pregnancy induces structural and functional modifications in offspring hippocampus, a brain region associated with learning and memory processes. Nevertheless, the exact mechanisms by which *in utero* diabetic environment affects the offspring hippocampus remain to be defined.

The main aim of the present thesis was to evaluate the impact of maternal diabetes on offspring's memory, evaluating first its' impact on early development and then uncover the underlying cellular and molecular alterations in the hippocampus that could be behind alterations found. Furthermore, evidences suggest that maternal diabetes impacts the offspring's brain in a gender-dependent manner, but few studies have been performed in both male and female offspring in order to establish a clear effect. Therefore we also intended to clarify possible gender-dependent impact of maternal diabetes on the offspring.

In order to evaluate the influence that foetal exposure to maternal diabetes could have during early postnatal development, a battery of tests was performed to assess reflexes, strength, and locomotion during the first postnatal weeks since altered performance in these tests could be suggestive of developmental sensory-motor system deficits. Maternal diabetes induced a delay in the development of reflexes, and impaired strength and locomotion during the first two postnatal weeks of both male and female offspring with a tendency for stronger effect on male offspring. The achievement of physical developmental milestones was also assessed and revealed, as well, a delayed development in both male and female offspring of diabetic dams. Furthermore, this longitudinal study showed impairments in weight gain of the offspring of diabetic dams of both genders.

Since the main aim of this thesis was to evaluate the impact of maternal diabetes on offspring memory in late infancy period, and since anxiety may affect cognition, we assessed anxiety-like behaviour in the open field (OPF) and elevated plus maze (EPM) tests. Locomotor activity was also evaluated in the OPF, as locomotor impairments may influence offspring performance in memory tests. No alterations were observed in locomotion in both female and male offspring of diabetic dams. Regarding anxious-like behaviour, a gender-specific impact of maternal diabetes was observed. Females demonstrated a tendency to explore less the central area of the OPF arena and entered fewer times EPM open arms suggesting an anxious-like behaviour, whereas no changes were observed for males. To uncover the impact of maternal diabetes in offspring memory we performed the novel object recognition (NOR) test. Even though we were unable to carry

out a gender differential analysis, results strongly indicate that maternal diabetes negatively impacts offspring memory.

To investigate the underlying cellular and molecular mechanisms that may be responsible for the cognitive deficits, we started by evaluating the structural organization of hippocampal subregions by cresyl violet staining. No differences in the thickness of the hippocampal subregion nuclear layers were detected suggesting that maternal diabetes did not induce neuronal loss. Furthermore, we evaluated the levels of proteins involved in synaptogenesis namely PSD-95, synapsin-1, synaptophysin and KIF1A. Maternal diabetes did not induce changes in the protein levels of these synaptic proteins nor in the levels of VGluT-1 as an indicator of glutamatergic synapses. AMPA receptor subunit levels were also evaluated since these receptors are important for synaptic plasticity. We observed a tendency for decreased total levels of GluA1 in the hippocampus of female offspring of diabetic dams and a tendency for increased synaptic GluA2 in the hippocampus of male offspring. These changes may functionally impact neuronal transmission and synaptic plasticity, which are important features in the context of memory processes.

This work highlights the importance of fully understanding the deleterious effects of maternal diabetes during brain development, as well as their long-term impact on neurobehavioral circuits. Furthermore, this study also demonstrates the importance of studying gender differences in response to insults during neurodevelopment.

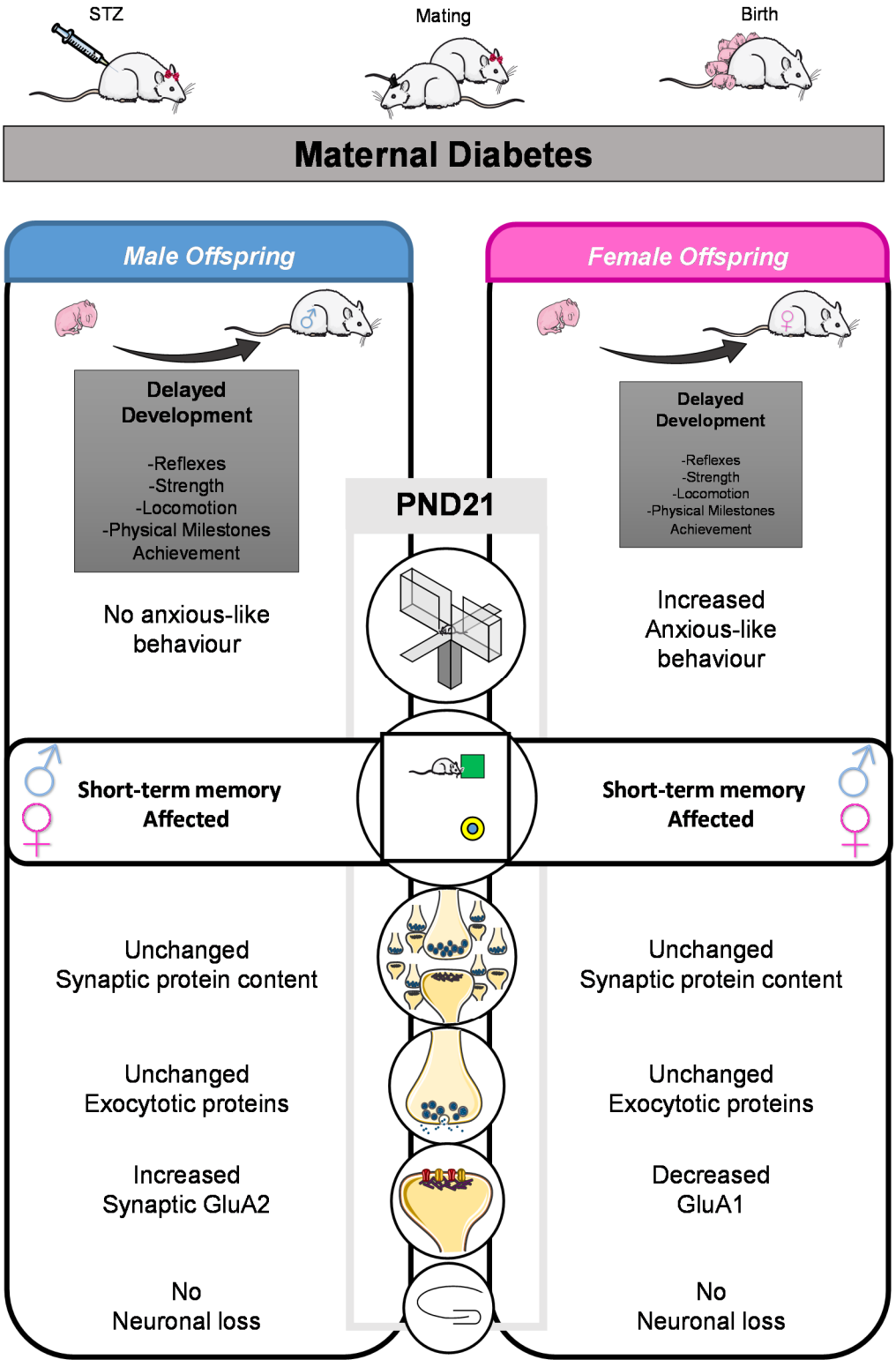
A better understanding of these issues will allow delineating future therapeutic strategies to prevent and treat neuronal dysfunction underlying memory and cognitive deficits of the offspring of diabetic mothers.

Keywords: Maternal diabetes, Neurodevelopment, Memory, Synaptogenesis, Gender differences

Graphical Abstract

Graphical abstract depicting the animal model and the main results presented in this thesis.

The left panel presents the main results regarding male offspring of diabetic dams whereas the right panel presents the results regarding female offspring.



Chapter 1

1. Introduction

1. Introduction

1.1. Brain Development

1.1.1. Overview of neuronal development

The human neurodevelopment starts during the third week of gestation with the differentiation of neural progenitor cells. Around gestational week 6, neuron production begins and is extended until week 16, through a process called neurogenesis (Stiles 2011). Nevertheless, in specific brain subregions, as the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles, neurogenesis continues during adulthood (Ming & Song 2011). As they are produced, neurons start migrating to different brain areas where they begin to make connections with other neurons. To become fully integrated into the neuronal network, neurons develop neuronal processes (axon and dendrites). Neurons connect with each other through synapses which allow transmission of electrochemical information (Brown et al. 2001). The process of synaptic formation is called synaptogenesis and starts during gestation continuing after birth (Stiles & Jernigan 2010).

The maturation of neuronal networks is achieved by a period of exuberant formation of synapses followed by neuronal apoptosis and synaptic pruning (elimination of excessive synapses). These events are naturally occurring and nonpathological, playing an essential role in the establishment of the complex network of the developing brain. By the end of the gestational period, major fibre pathways are completely formed (Stiles & Jernigan 2010). The brains' functional architecture is therefore established during development, but interactions with the world keep continuously sculpting the brain (Sanes et al. 2006).

Humans and rodents have different ontogeny regarding neural development. While in humans most of the neuronal maturation occurs prenatally, in rodents there is considerable neural development postnatally. However in terms of proliferation and migration, there is a parallelism between rodents and humans for the pattern of regional development with the exclusion of the time-scale difference (Rice & Barone 2000; Semple et al. 2013).

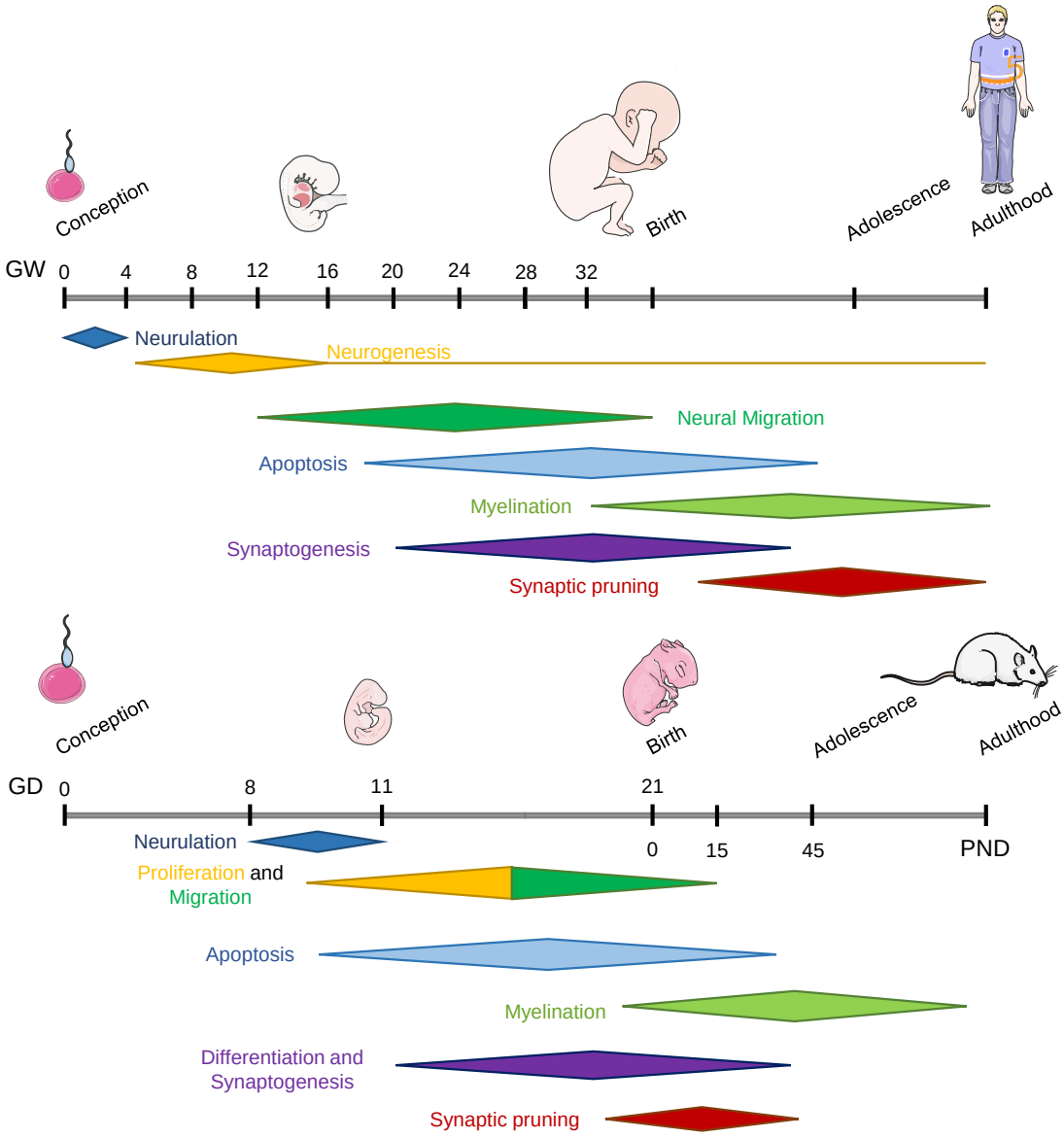


Figure 1. Comparative representation of rat and human brain developmental timeline | Representation of neurodevelopmental processes in rat and human, during gestation (gestation day – GD / gestation week - GW) and after birth (postnatal day - PND) up to adulthood (not to scale). Adapted from Semple et al. 2013 and Rice & Barone 2000.

1.1.2. Synaptogenesis

Synaptogenesis is the processes of formation of synaptic connections between neurons. Most synapses form during pre- or early postnatal development and in this initial phase of development, synaptogenesis occurs in the absence of neuronal depolarization and neurotransmitter release. Synapses survive only from days to months and must be renewed throughout life span. Synapse formation, therefore, continues into adulthood and is highly governed by neuronal activity allowing very active synapses to facilitate the formation of additional synapses (Lardi-Studler & Fritschy 2007; Wurtman et al. 2009).

Synapse formation in the hippocampus can be initiated by the approach of both postsynaptic dendritic spine and presynaptic terminal (Toni et al. 2007). Cell adhesion molecules are described to contribute to the appropriate matching of the synapse, guiding axons toward the correct target, forming the initial contact between the synaptic sites and recruiting proteins for functional synapse formation (Washbourne 2004; Lardi-Studler & Fritschy 2007). Right after initial contact between the axon and the dendrite, synaptic vesicles begin to accumulate at the presynaptic site (Smith et al. 2000) followed by postsynaptic site differentiation. For proper synaptic function all the components of the synapse must be precisely aligned and clustered across the synaptic cleft and the postsynaptic receptors. Scaffolding and signalling proteins must be sorted according to presynaptic terminal nature (Garner et al. 2002; Bury & Sabo 2016).

Several proteins have been proved critical to synaptogenesis in the presynaptic terminal (Figure 2). KIF1A, a microtubule-based molecular motor protein, transports synaptic vesicle precursors containing synaptic proteins namely synaptophysin and synaptotagmin (Okada et al. 1995; Yonekawa et al. 1998) and its' role has been implicated in synaptogenesis. Since an overexpression of KIF1A in cultured hippocampal neurons leads to an increase of synaptophysin (presynaptic marker) co-localisation with post synaptic density (PSD) 95 (postsynaptic marker), it is suggested that KIF1A promotes synaptogenesis via formation of presynaptic boutons (Kondo et al. 2012). Synaptophysin appears as a small synaptic vesicle protein suspected to be involved in regulating synaptic vesicle exocytosis, appearing prior and parallel to synapse formation and is therefore considered a good marker of synaptogenesis (Vafaei-Nezhad et al. 2016). Also, synapsin-1 neuronal phosphoprotein, plays a role in regulation of various stages of neurodevelopment being involved in the regulation of synaptic vesicle clustering in mature nerve terminals, playing therefore, a role in synaptogenesis (Chin et al. 1995).

During synaptogenesis, besides presynaptic vesicle clustering, the post synaptic terminal must be properly assembled. The postsynaptic terminal of the excitatory synapse is characterised by an electron-dense matrix, the postsynaptic density that contains molecules involved in glutamate receptor targeting and trafficking. The PSD-95 is a scaffolding protein present at postsynaptic densities, clustering glutamate receptors at the membrane, maintaining high local concentration and coupling them to downstream signalling molecules. PSD-95 has also been proposed to affect synapse maturation and stabilisation, impacting on synaptogenesis process (Sen et al. 2016).

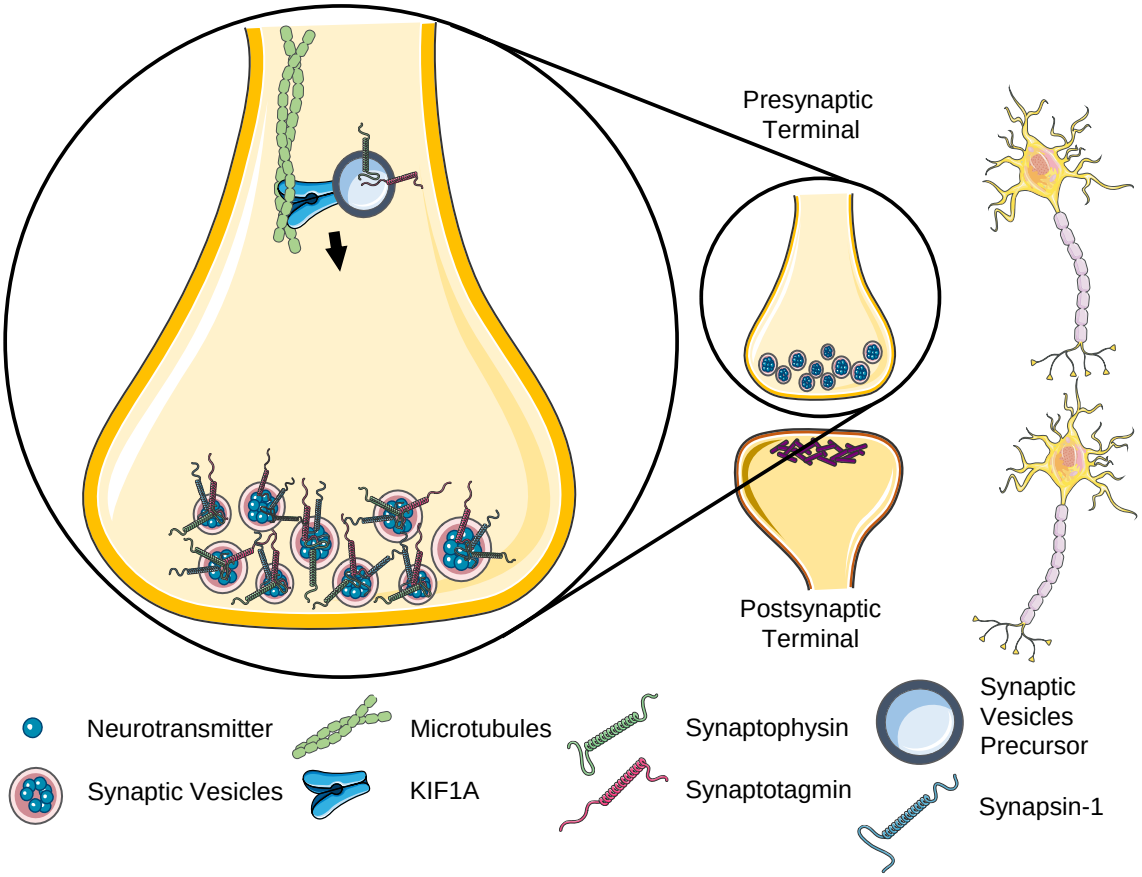


Figure 2. Representation of the synapse | Depiction of the alignment of the presynaptic terminal's active zone and the postsynaptic terminal.

1.2.3. Synaptic transmission

The majority of synapses do not physically connect, instead, the presynaptic and postsynaptic components communicate via secretion of molecules from the presynaptic component, the neurotransmitters, that bind to receptors in the post synaptic specialisation. This type of synapse is called the chemical synapse (Purves et al. 2008). The major excitatory neurotransmitter is glutamate, which is stored in synaptic vesicles by the action of vesicular glutamate transporters (VGluT). There are three isoforms, VGluT1, VGluT2 and VGluT3 the first two are selectively expressed in glutamatergic presynaptic terminals whereas the last one is in glutamatergic neurons, as well as, non-glutamatergic neurons and astrocytes. VGluT1 accounts for approximately 80% of total vesicular glutamate uptake in the CNS being expresses predominantly in the cerebral and cerebellar cortex and hippocampus, whereas VGluT2 in the subcortical neurons, though both isoforms may be co-expressed in the same synaptic terminal (Benarroch 2010). To reach the postsynaptic specialisation molecules must cross an interval of extracellular space called synaptic cleft.

The process by which the information is transmitted in the synapse is called synaptic transmission (Figure 3). Transmission at chemical synapses results of an elaborate sequence of events which starts when an action potential arrives at the axon terminal. Action potential triggers Ca^{2+}-channel opening, leading to an increase in the presynaptic terminal concentration of Ca^{2+}. In turn, Ca^{2+} increase allows synaptic vesicles that contain stored neurotransmitters, to release their content into the synaptic cleft. Synaptic vesicle fusion with the presynaptic plasma membrane occurs at a specialised region, the active zone. Following exocytosis, the neurotransmitters diffuse in the synaptic cleft and bind to specific receptors on the postsynaptic neuron's membrane. Postsynaptic receptors are clustered opposite to the active zone in the post synaptic density and induce a current flow that alters conductance and membrane potential that increase or decrease the neuron's probability to fire an action potential and propagate the signal (Purves et al. 2008; Südhof 2014).

Two main opposite systems of neurotransmission exist in the brain representing the vast majority of synapses in the brain, the excitatory neurotransmission mediated by ionotropic glutamate receptors (N-methyl-D-aspartate-NMDA; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-AMPA and Kainate receptors), and the inhibitory one mediated by GABA and glycine receptors (Lardi-Studler & Fritschy 2007)

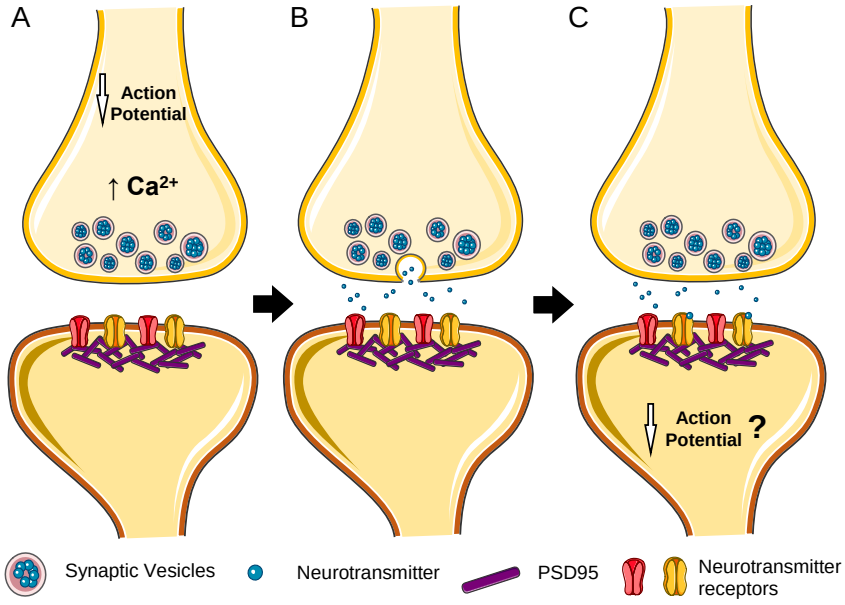


Figure 3. Schematic synaptic transmission | (A) An action potential arrives to the presynaptic terminal triggering the opening of Ca^{2+} channels that lead to an increase in presynaptic terminal concentration of Ca^{2+}. **(B)** Increase of Ca^{2+} leads to the fusion of synaptic vesicle with the plasma membrane and to the release of neurotransmitters. **(C)** Neurotransmitter cross the synaptic cleft and bind to specific receptors inducing current flow that can lead to the firing of an action potential.

In the hippocampus, glutamatergic synapses form at a later stage of development than GABAergic synapses and contain only NMDA receptors functionally “silent” (Lardi-Studler & Fritschy 2007). Functional AMPA receptor-mediated currents that provide main excitatory drive, in mature circuitry, appear towards the end of the first postnatal week in the rat (Lardi-Studler & Fritschy 2007; Ben-Ari 2002).

1.2. Hippocampus

The hippocampal formation is a brain structure located at the medial temporal lobe that belongs to the limbic system. It has a dual function according to their dorsal or ventral anatomic location. The dorsal hippocampus is implied in mediating spatial, working, contextual and recognition memory whereas the ventral one is related to stress and emotion (Koss & Frick 2017; Fanselow & Dong 2010).

In the human brain, the hippocampus develops prenatally during the third trimester whereas in the rat, brain development of the hippocampus is perinatal (Rice & Barone 2000). Anatomically, the hippocampus is distinguished as a zone where the cortex narrows into a curled up tight structure, shaped like an S, densely packed with neurons and organized in layers. This structure is comprised of 4 cortical regions: the dentate gyrus (DG); the hippocampus proper consisting in 3 subfields, the *Cornu Ammonis* (CA)1, CA2 and CA3; the subicular complex subdivided in the subiculum, *presubiculum* and *parasubiculum*, and lastly the entorhinal cortex (Amaral & Witter 1989).

The fields of the hippocampus are linked by largely unidirectional and mostly excitatory connections. The entorhinal cortex serves as the main “interface" between the hippocampus and other parts of the brain. It provides major input to the dentate gyrus via the perforant path. The dentate gyrus’ granular cells project via mossy fibres to the hippocampus’ CA3 field. Pyramidal cells in the CA3 field give rise to collateralized axons that terminate within CA3 as associational connections, and also provide the major input to the CA1 field pyramidal cells of the hippocampus, the Schaffer collaterals. The highly organized structure that is the hippocampus (Figure 4) offers a good model system for neurophysiology studies (Amaral & Witter 1989; Amaral et al. 2007; Deng et al. 2010).

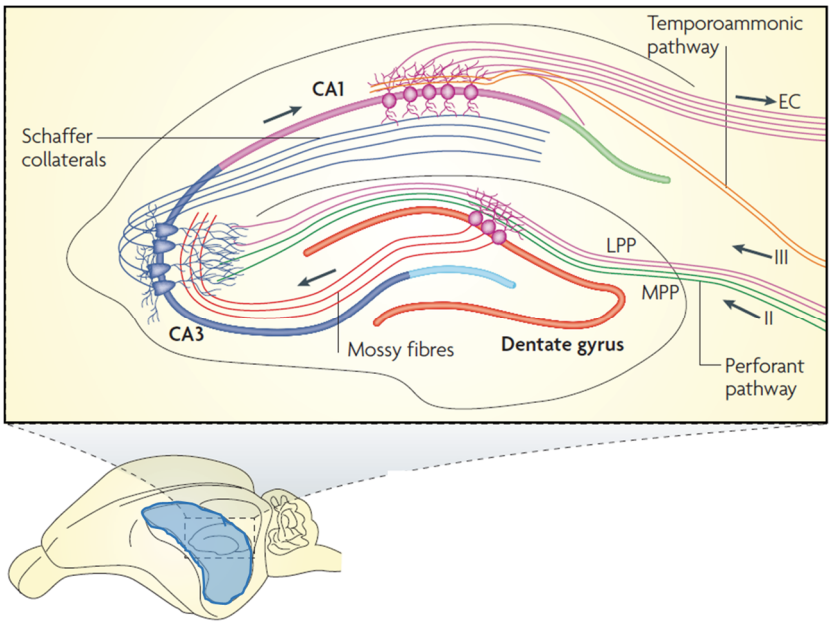


Figure 4. Schematic representation of the rat hippocampus and its' connectivity | Hippocampus is located in the medial temporal lobe. Its' structure is shaped like an S and the hippocampal subfields are linked by largely unidirectional connections that are mainly excitatory. Adapted from Deng et al. 2010.

1.3. AMPA Receptors

The AMPA receptors are tetrameric ion channels that mediate the majority of fast excitatory synaptic transmission in the brain and play an important role in synaptic plasticity (Nakagawa 2010). AMPA receptors are composed of multiple types of assemblies of GluA1-4 subunits. The differential subunit expression adds considerable functional diversity as different composition results in different channel kinetics, ion selectivity, receptor trafficking, contributing therefore to different synaptic and extrasynaptic localisation (Greger et al. 2017; Jacobi & von Engelhardt 2017).

The presence or absence of GluA2 subunit in the functional receptor is a major regulator of its properties. In the brain most of GluA2 appears as a RNA edited form, resulting in a change from glutamate to arginine (Q/R) at position 607 (150) (Henley & Wilkinson 2016). The amino acid charge alteration in the GluA2 subunit will result on AMPA receptors channels that contain this edited GluA2 to have a pore less permeable to Ca^{2+}. Additionally, this edited GluA2-containig AMPA receptors are not blocked by intercellular polyamines and have reduced single-channel conductance. Moreover, Q/R editing at position 607 also results in altered GluA2-containing receptors trafficking properties (Henley & Wilkinson 2016; Greger et al. 2017).

The pore subunit constitution, even being the primary contributor for AMPA receptors' property diversity, is not the only one. Alternative RNA splicing, post-translational modifications and association of several auxiliary proteins, such as stargazin/TARPs, cornichon and CKAMP44, among many others, alter receptors' properties and are essential sources of modulation of synaptic transmission (Greger et al. 2017).

1.3.1. AMPA receptor functional characteristics

Learning and memory are described to depend on long-lasting changes in synaptic plasticity and synaptic strength. Long-term potentiation (LTP), specifically in the hippocampus, is part of a process that strengthens synaptic transmission and is therefore widely considered the major cellular mechanism that underlies learning and memory (Kandel et al. 2000)

In order to strengthen synaptic transmission, depending on the type of LTP, changes may be induced both in pre- and postsynaptic terminals (Kandel et al. 2000). In the postsynaptic terminal, after NMDA receptor activation, synaptic strength is achieved by recruitment and insertion of clusters of AMPA receptors from the intracellular polls stored in recycling endosomal vesicles (Shimshek et al. 2017).

AMPA receptor currents are broadly mediated by GluA1/GluA2 and only slight by GluA2/GluA3 AMPA receptors (Shimshek et al. 2017). The GluA4 subunit is not involved in AMPA mediated transmission in the adult mice, only while synaptic connectivity is under formation (Luchkina et al. 2017). GluA2/GluA3 exist in low levels but are sufficient to maintaining basal synaptic transmission. Furthermore, the GluA1 subunit is necessary for the extra synaptic pools of AMPA receptors which are widely composed of GluA1/GluA2 and are actively translocated to potentiated synapse upon LTP induction (Shimshek et al. 2017)

1.3.2. Subunit expression during development

In early development, GluA4 homomers are preferential inserted into silent synapses at PND5-PND7 through a process which is activity and NMDA-dependent (Zhu et al. 2000). GluA4 homomers are subsequently replaced with GluA2 containing receptors by a process that maintains synaptic strength (Henley & Wilkinson 2016).

After birth, GluA2 expression is low, as compared to GluA1. AMPA receptors in this early life period are GluA2-lacking and therefore Ca^{2+}-permeable. After the second postnatal week many synapses contain GluA2-lacking Ca^{2+}-permeable AMPA receptors

which are exchanged for GluA2-containing Ca^{2+}-impermeable AMPA receptors (Pellegrini-Giampietro et al. 1992).

The predominant GluA1 expression is highly developmentally restricted, after birth GluA1 expression is higher than GluA2 but by PND14 almost all AMPA receptor positive synapses express GluA2 (Monyer et al. 1991).

At PND21 another AMPA receptor subunit shift in composition is observed as GluA3 levels increase and GluA1's decline. AMPA containing the GluA3 subunit in its composition show reduced deactivation and desensitisation compared to GluA1-containing AMPA receptors (Suzuki et al. 2008; Henley & Wilkinson 2016) this could account for the developmental increase in duration of AMPA receptor's responses, postsynaptic excitability and for the reduction LTP threshold (Henley & Wilkinson 2016).

1.4. Developmental Behaviour

1.4.1. Development of the sensory-motor system in the rat

Animals rely on their sensory-motor system to perceive the environment. While in humans most of sensory development occurs *in utero,* in the case of rodents there is still considerable neurodevelopment after birth (Grubb & Thompson 2004; Rice & Barone 2000). Motor function and sensory systems including visual, auditory and somatosensory reside in specific areas of the neocortex, both in rats and humans (Kolb & Tees 1990; Heimer 1995). In terms of sensory systems, the biggest difference between humans and rodents reside in the fact that humans highly rely on visual cues, whereas rodents are mostly dependent on auditory and olfactory cues. Consequently, the brains areas that are devoted to each sensory system also reflect this difference (Rice & Barone 2000).

Gottlieb (1971) compiled data from different species and was able to detect that the onset of function for the sensory systems was highly conserved following a specific order (Figure 5). The first to develop was the vestibular system, then olfactory, tactile, auditory and lastly visual, although some species developed it before birth/hatching and others only after it (Kail & Spear 1984; Gottlieb 1971).

The vestibular system locates in the inner ear and provides information related to gravity force, angular and accelerative movements. Vestibular perceptions contribute to the maintenance of head and body orientation being also important for coordinative movements. It's among the first of the sensory systems to become functional (Kail & Spear 1984). In rats, at birth, this system seems to be immature since animal display a rudimentary righting reflex. In fact, at PND1-PND2 peripheral and central vestibular neurons show some response while at PND8 it already resembles that of the adult animal (Wills et al. 2014).

Neonatal rat's olfactory perception is described as being the following to develop. It is essential for pup survival, important for suckling, for home nest and mother recognition. In fact, rats demonstrate a preference for their mother at PND2, rudimentary sniffing is detected at PND4 and the adult-like sniffing behaviour is only detected at PND10-PND11 (Wills et al. 2014; Kail & Spear 1984).

Tactile exploration is believed to be mediated by active whisking (repetitive and rapid sweep back and forth of the facial macrovibrissae) and is first observed at PND4 but adult-like whisking behaviour emerges at PND10-PND13 (Wills et al. 2014).

The auditory system develops after birth as well. At PND11-PND12 auditory action potential response is observed in auditory nerves and the opening of rat meatus is at PND11-PND13, which is when animals start showing orientation towards auditory stimulus (Wills et al. 2014).

The last of the sensory systems to develop is the visual system. Eye opening happens about PND13-PND15 and evidence seem to indicate that visual cortex starts responding to stimulus at PND16, but visual acuity, for example, only becomes adult-like at PND45 (Fagiolini et al. 1994) denoting a larger period of maturation comparing with the development of the other sensory systems (Wills et al. 2014).

In terms of motor skills development (Figure 5), firstly emerges the skill for animals to right themselves at PND0, between PND3-PND7 animals acquire pivoting-like movement. This is the first organized locomotor behaviour, a movement characterized forelimb activity and hindlimb inactivity. At one week of age, animals acquire crawling-like movement where hindlimbs are still not contributing for movement. Properly coordinated walking with the full use of hindlimbs is not observed until PND14. By PND 21 animals show acquisition of a large range of complex motor skills comparable to adult movement (Wills et al. 2014).

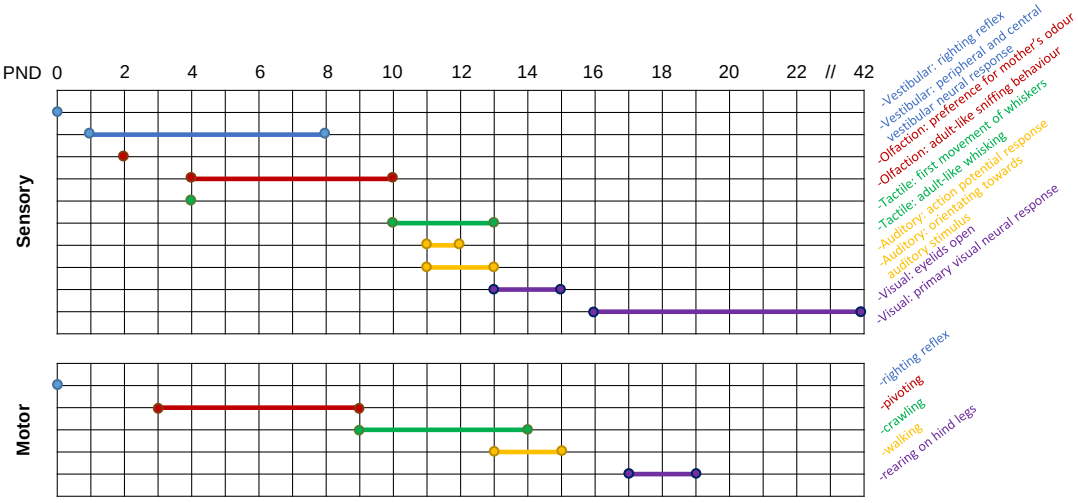


Figure 5. Summary timeline of sensory and motor development in the rat | Vertical lines indicate the age of the animal (postnatal day - PND), each horizontal line represents the development of a particular sensory function or motor ability. Between PND22 and PND42 the timeline was graphically shortened. Each colour represents either a different sensory system or a different motor skill. Adapted from Will et al. 2014.

1.4.2. Development of spatial behaviour in the rat

The first sign of rats' spontaneous expression of movements through space is the ability for pups to return to their nest if separated from their mother or littermates. Orientation to the nest is described to appear around PND3-PND8 but the ability to reach home nest (homing to nest) will not develop until PND8 since crawling ability develops at the same time (Altman & Sudarshan 1975; Wills et al. 2014).

Adult rats have an innate drive to explore new environments which is a hippocampal driven behaviour (Berlyne 1966; Save et al. 1992). This drive for spontaneous exploration and curiosity also emerge during early development (Bolles & Woods 1964) being mostly centred between PND13-PND21 even though maturation of such like behaviours (exploration of open field and of objects) still happens at later ages PND30-PND90 (Figure 6) (Wills et al. 2014)

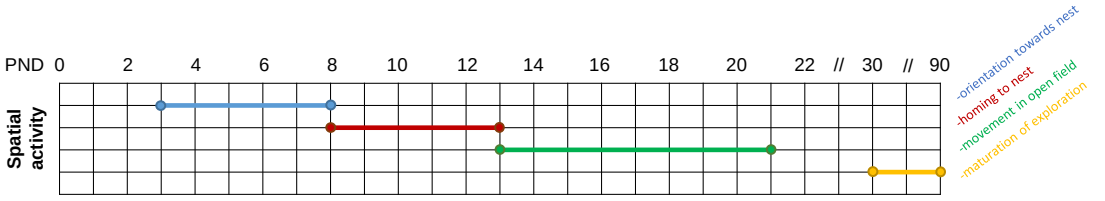


Figure 6. Summary timeline of spatial behaviour development in the rat | Vertical lines indicate the age of the animal (postnatal day - PND), each horizontal line represents the development of a particular spatial behaviour activity. Between PND22 and PND90 the timeline was graphically shortened. Each colour represents a different spatial behaviour. Adapted from Will et al. 2014

1.5. Diabetes in Pregnancy

1.5.1. Diabetes

Diabetes mellitus is a group of metabolic diseases characterized by chronic hyperglycaemia associated with insulin action and/or secretion deregulation that leads to metabolic system abnormalities. Diabetes can generally be classified into type 1, type 2 and gestational. Type 1 diabetes is also called insulin dependent diabetes or juvenile-onset diabetes. It is characterized by pancreatic β-cells destruction, either by autoimmune or idiopathic related mechanism, followed often by a complete insulin deficiency. Type 2 diabetes is referred to as non-insulin-dependent or adult-onset diabetes. It is characterized by insulin resistance and also by a degree of insulin secretion deficiency caused mainly by lifestyle habits like overeating and lack of exercise. Gestational diabetes is a form of diabetes that is transient and affects non-diabetic women during pregnancy. It occurs in 4% of pregnancies and is due to placental hormones that may block mother's insulin action leaving mothers insulin resistant during pregnancy (Seino et al. 2010; American Diabetes Association 2010).

1.5.2. Impact on the developing brain

The adaptation that foetus undergoes due to suboptimal intrauterine conditions creates long-term health consequences. Barker in 1993 referred to it as the "foetal origins of adult disease" (Barker et al. 1993). So in diabetes, the intrauterine environment has been evidenced to create an adverse condition for foetal growth and development and is proposed to have consequences later in life (Van Assche et al. 2001). This early in life susceptibility for developmental abnormalities has been observed not only *in utero* but also after birth (Holemans et al. 1999).

In a study by Aerts et al. (1990), it was described the impact of maternal diabetes in the offspring's insulin production. In an animal model, they verified that destruction of maternal pancreatic β-cells left foetus exposed to a very high glucose level that leads to β-cell hyperactivity. After a temporary increase in insulin release, the continued insult leads pancreatic β-cell islets to become disorganized and exhausted during development, thus provoking hypoinsulinemia in the foetus (L Aerts et al. 1990).

Besides this alteration, in maternal diabetes, there is a higher probability for developmental abnormalities such as congenital malformations, incomplete sacral ossification, caudal dysgenesis and also abnormalities in musculoskeletal, cardiovascular and central nervous systems. (Weintrob et al. 1996)

During development, the brain is particularly susceptible to developmental alterations and altered glucose levels may have long-term neurologic effects if not controlled (ter Braak et al. 2002). This may explain the finding that offspring of diabetic mothers tend to have poor academic performance (Rizzo et al. 1997; Akyol et al. 2003). Also, other neuropsychological alterations have been reported as poorer intelligence (DeRegnier et al. 2000; Nelson et al. 2000), altered psychomotor skills (Ornoy et al. 1998; Rizzo et al. 1995), impaired sensory function (Stenninger et al. 1998) as well as decreased levels of attention (Ornoy et al. 1998; Stenninger et al. 1998), all of which associated with poor maternal diabetes control.

Offspring of diabetic mothers have been hypothesized to be prone to hippocampal damage and impaired memory recognition. Even though hippocampal damage is challenging to be assessed in human, correlations might be a possibility. In this manner, infants born to diabetic mothers submitted to explicit memory performance tests have shown memory performance deficits (Deboer et al. 2005). Evaluation of electroencephalograms' event-related potentials, which may be used to assess neural pathways in cognitive neuroscience, have also been used to evaluate infants born to mothers with diabetes. Evidence of altered event-related potentials profiles has been brought to light in infants born to diabetic mothers, during recognition related tests

(DeRegnier et al. 2000; Nelson et al. 2000). This supports the idea that maternal diabetes might be interfering with the hippocampus.

Animal models have also been a focus of study in order to unravel the impact of maternal diabetes on the offspring. Ramanathan et al. (2000) showed that that offspring of diabetic mothers, regardless of gender differences, display hyperactivity in the open field and anxious-like behaviour in the elevated plus maze (EPM) tests (Ramanathan et al. 2000). On the other hand, Kinney et al. (2003) performed gender differential behavioural analysis and did not see any differences in terms of anxious-like behaviour in the EPM. The authors' cognitive analysis of the impact of maternal diabetes in the offspring showed that only the female diabetic offspring showed deficits on the Lasheley III maze and in the inhibitory avoidance task, that test long-term memory and learning. Whereas while testing for deficits in short-term/working memory in the 12-arm radial arm maze, no differences were found in both male and female offspring of diabetic mothers (Kinney et al. 2003). In an animal model of gestational diabetes, induced at GD13, Chandna et al. 2015, found that the male offspring of diabetic mothers dwell more on the open arms than control ones, appearing to be disinhibited. Furthermore, these authors could also detect an increased exploration of novel object displacement on the novel object recognition (NOR) not showing impaired cognition and rather an increased exploration interest. However, in a protocol for behaviour flexibility, Chandna et al. (2015) showed that the diabetic offspring had the ability to learn but when faced with a paradigm change, where a new behavioural strategy had to be adopted, male offspring had a poorer performance in the task (Chandna et al. 2015). These studies, even though not showing the same outcome, support the idea that maternal diabetes may impact hippocampal related behaviour. Additionally, the afforementioned studies also suggest that male and female offspring may have different susceptibility to maternal diabetes effects.

Several cellular and molecular mechanisms may underlie the impact of maternal diabetes on offspring brain. Insulin-like growth factor-I (IGF-I) is important for brain growth and development. It stimulates proliferation of neuronal progenitor cells, synaptogenesis and induces differentiation and survival of neurons. Hami and colleagues found that, in a rat model, maternal diabetes had an impact on IGF-1R expression levels and distribution, both in the cerebellar cortex and the hippocampus of offspring. In the cerebellar cortex, they reported that male offspring had increased IGF-1R positive granule cells at PND0 and decreased at PND14 (Hami, Vafaei-Nezhad, Haghir, et al. 2016). In the hippocampus, the IGF-1 expression levels were found increased at PND0 but not at two weeks, whereas the protein levels at PND0 were not altered but at PND7 were decreased (Hami et al. 2013). Furthermore, in another study, the same group also reported a significant reduction in the thickness and volume in the cerebellar external granule, molecular and internal granule

layers in the male offspring, in several early postnatal timepoints (Hami, Vafaei-Nezhad, Ghaemi, et al. 2016). Rozi et al. (2015) also reported a neurotoxic effect of gestational diabetes, induced at GD1 in the cerebellar Purkinje cells, further proving the strong impact of uncontrolled diabetes during pregnancy (Razi et al. 2015). Most of these effects were prevented through insulin administrations to diabetic dams, demonstrating the importance of strict glycaemic control during pregnancy.

Golalipour et al. (2012) also reported, in a model of gestational diabetes, a reduction of pyramidal cells at the CA3 and CA1 subfield regions of the hippocampus in male offspring, suggesting a cause of disabilities in learning and memory in reported in humans (Golalipour et al. 2012). Decreased synaptophysin levels were detected at the CA1, CA3 and DG at PND7 and PND14 in the offspring (Vafaei-Nezhad et al. 2016), as well as decreased synaptophysin mRNA levels at the same timepoints suggesting a possible impairment in synaptogenesis (Vafaei-Nezhad et al. 2016).

Further studies are needed for a better understanding of the mechanisms responsible for the effects of maternal diabetes on cognitive development of the offspring. Clarification of such mechanisms could allow delineating future therapeutic strategies to prevent and treat neuronal dysfunction underlying memory and cognitive deficits of the offspring of diabetic mothers.

Chapter 2

2. Rationale and Aims

2. Rationale and Aims

Maternal diabetes has been described to provide an *in utero* aversive environment for foetal development that has been associated with psychomotor, sensory and cognitive deficits in children of diabetic mothers.

Experimental studies have suggested that maternal diabetes impairs the formation of synapses, affecting hippocampal structure and inducing learning and memory deficits in the offspring (Vafaei-Nezhad et al. 2016; Vuong et al. 2017; Golalipour et al. 2012). Furthermore, diabetes-induced hippocampal changes have been suggested to be gender-specific (Kinney et al. 2003).

Taking into account previous data, the main aims of this thesis were:

- To assess the impact of maternal diabetes on offspring achievement of developmental milestones during early postnatal life.
- To evaluate the impact of maternal diabetes on offspring memory in late infancy.
- To uncover the underlying cellular and molecular alterations in the hippocampus that could account for the memory deficits that are reported in maternal diabetes, namely 1) by evaluating the levels of key proteins involved in synaptogenesis; 2) by analysing the content of proteins important for glutamatergic neurotransmission and synaptic plasticity 3) by assessing the structural integrity of hippocampal subregions nuclear layers.
- To evaluate if maternal diabetes-induced hippocampal changes are gender-specific.

Chapter 3

3. Methods

3. Methods

3.1. Animals

Procedures involving animals were performed in agreement with the EU guidelines for the use of experimental animals (EU Directive 2010/63/EU). Animals used were acquired from the Faculty of Medicine animal housing. Mating females were randomly assigned to control and diabetic groups. Animals were housed in temperature and humidity controlled environment with 12-hour cycles of light/dark (lights on at 8:00 h) and had *ad libitum* access to water and food.

3.1.1. Experimental design

Female Wistar Han rats aged 8 weeks were divided into 2 experimental groups: control and diabetic. Type 1 diabetes was induced by a single intraperitoneal (ip) injection of streptozotocin (STZ) 45 mg/Kg (in 100 mM sodium citrate buffer pH 4.5) to overnight fasting female rats. This STZ dose has been described to induce moderate diabetes (Vafaei-Nezhad et al. 2016). After STZ injection, animals were again given *ad libitum* access to food, and drinking water was replaced with 5 % (m/V) sucrose solution for 1 day to attenuate the sudden hypoglycaemic state induced by a large quantity of insulin release due to pancreatic β-cells death (Furman 2015). Glycaemia was measured before and 2 days after STZ injection with a glucometer (Ascensia ELITE™, Bayer Corporation, Mishawaka, IN) by pricking the tip of the tail. Animals were considered diabetic when blood glucose levels exceeded 250 mg/dL. After 1 week of diabetes, females were mated with aged-matched control male Wistar rats for a 3 days mating period.

After mating, female dams weight and glycaemia were monitored regularly till they gave birth (gestation day 21; GD21). After birth neonatal animals were weighted at postnatal day (PND) 0, PND7, PND14 and at PND21 and blood glycaemia insulin levels were quantified at PND21.

To assess blood glucose and insulin levels at PND0 and PND7, an additional subset of animals was used since animals must be sacrificed to collect enough blood to assess this parameter.

A battery of infancy behavioural tests, from PND5 to PND17, was performed to evaluate reflex and motor development. Namely Surface Righting Reflex (PND5-PND10), Negative Geotaxis Reaction (PND5-PND14), Cliff Aversion (PND5-PND10), Wire Suspension (PND10-PND14), Locomotion (PND5-PND14) and Nest Seeking test (PND5-PND15) were performed to offspring born from control (CTRL) or diabetic (STZ) females.

Day of Eye Opening (PND12-PND17) and earing ability, through Auditory Startle Reaction (PND11-PND14), were assessed as physical developmental milestones.

Late infancy behavioural tests (PND19-PND21) namely Open Field (OPF), Elevated Plus Maze (EPM), and Novel Object Recognition (NOR) tests were performed as described in the next subsection. At PND21 all animals were sacrificed, samples were harvested for all subsequent analysis. Schematic representation that summarises experimental design can be consulted (Figure 7).

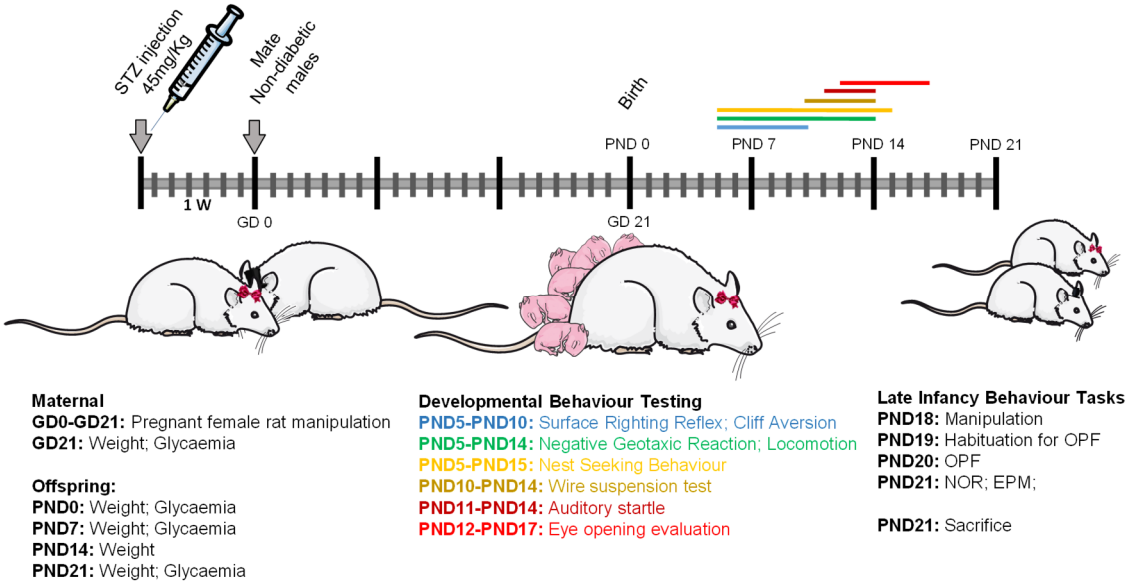


Figure 7. Schematic representation of the experimental design | Female Wistar rat dams were injected with STZ (45mg/Kg, ip) and were mated with non-diabetic males 1 week after STZ injection. At GD21 females' weight and glycaemia was measured. Offspring were weighted and glycaemia measured at PND0, PND7, PND14 and PND21. Between PND5-PND17 the offspring were submitted to a battery of developmental behaviour testing. From PND18-PND21 the offspring were submitted to late infancy behaviour tasks. At PND21 offspring were sacrificed and samples were collected for further processing.

3.2. Behaviour

3.2.1. Developmental behavioural testing

Neonatal pups from CTRL and STZ dams were submitted to a battery of tests from PND5 to PND17 for assessment of developmental behaviours, testing for motor and reflex development. All neonatal behaviour was performed on the light phase under dim white light from 12:00h to 16:00h. Day of eye opening and earing ability was monitored as a physical development milestone parameter. Pups were only separated from their dams for a short period of time to prevent pup's rapid loss of body heat and hunger/separation issues. Each value for each pup was considered as an independent measure.

3.2.1.1. Surface righting reflex

Pups PND5-PND10 were placed on their back on a flat soft surface and were held down for 5s. The time, after release, taken by the animal to return to its four limbs was noted evaluating trunk control, vestibular system deficits. The cut-off time for this test was 5 s (Figure 8) (VanRyzin et al. 2016).

Figure 8. Representation of the surface righting reflex test | Rat pups PND5-PND10 were placed on their backs on a soft surface and were evaluated for the ability to turnover on the righting reflex test.

3.2.1.2. Negative geotaxis reflex

Animals PND5-PND14 were placed face-down on a platform with a 35-degree incline, covered with Eva Sponge to enable traction. The time the animal took to turn from the upside down position to a point where both forepaws were top oriented was evaluated as an indication of coordination, balance and vestibular input deficits. Cut-off time for this test was at 30 s (Figure 9). Animals that fell from the platform were given 3 tries after which a score of 30 s was attributed to non-performing animals (Fernández de Cossío et al. 2016; VanRyzin et al. 2016).

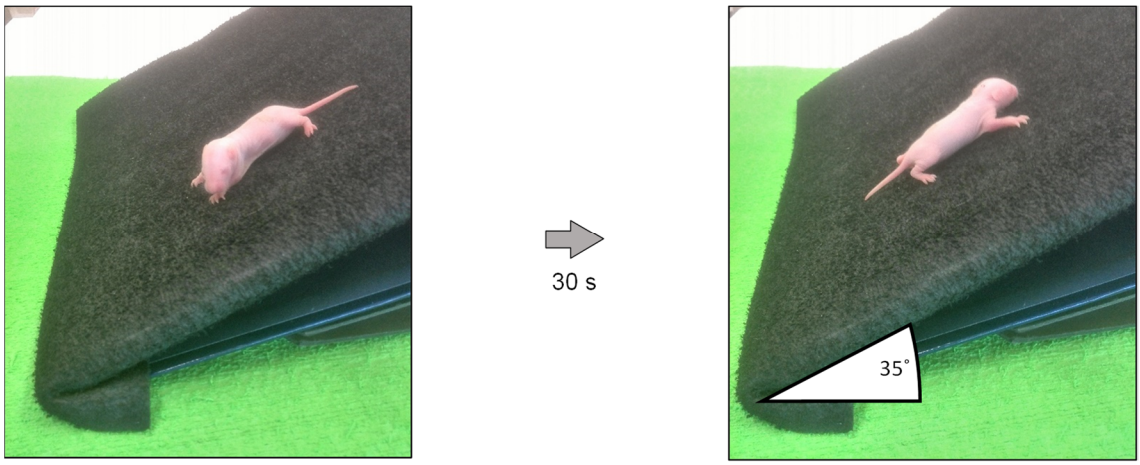


Figure 9. Representation of the negative geotaxis reaction test | Rat pups PND5-PND14 ability to turn from the upside down position and face upwards on a 35˚ incline plane was assessed on the negative geotaxis test.

3.2.1.3. Cliff aversion

Pups PND5-PND10 ability to retract from the edge of a cliff was assessed. Animals were placed on an elevated flat surface only with its snout and forepaw digits hanging over the edge. The time was counted for the pup to turn away from the cliff and move its paws and snout away from the edge, up to 30 s (Figure 10).

If within 30 s no response was seen, the test was finished. If the pup fell off the edge, a single additional trial was performed (VanRyzin et al. 2016; Feather-Schussler & Ferguson 2016).

Figure 10. Representation of the cliff aversion test | Rat pups PND5-PND10 ability to retract from the edge of a cliff was assessed on the cliff aversion test.

3.2.1.4. Wire suspension

The pup's forelimb strength was assessed through the wire suspension test for PND10-PND14 animals. The pups were placed against a horizontal wire rod (covered with satin thread) and were allowed to grasp it with both forepaws. This wire was stiffly strung above a padded drop zone. Time was scored after the release of the animal until it fell to the padded drop zone with cut off time at 10 s (Figure 11). Pups that fall immediately were given 3 trials (VanRyzin et al. 2016).

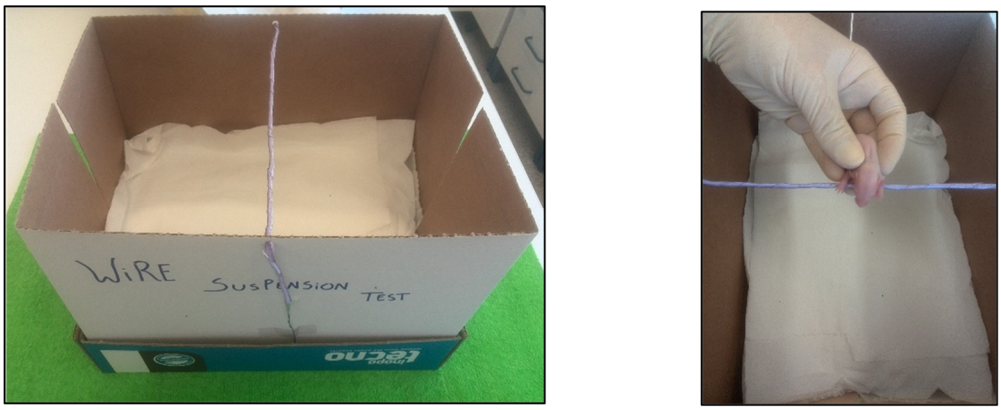


Figure 11. Representation of the wire suspension test | Rat pups PND10-PND14 ability to hang on a wire for 10 s was assessed on the wire suspension test.

3.2.1.5. Locomotion

Pup's PND5-PND14 locomotion was assessed placing animals on a flat surface in the centre of 13 cm diameter circle. The time was recorded for the animal to fully exit the arena with all four limbs within a 30 s time trial (Figure 12) (VanRyzin et al. 2016).

Figure 12. Representation of the locomotion test | Rat pups PND5-PND14 were placed on the centre of a 13 cm diameter circle and time was recorded for them to fully exit the arena with all four limbs within a 30 s time trial.

3.2.1.6. Nest seeking

Pups PND5–PND15 ability to discriminate its' nest bedding by olfaction was determined through the Nest Seeking Test. For this test, we used a rectangular arena

(26x14 cm) divided into 3 compartments: the centre where the pup was placed; the home bedding goal on one side where there was home nest bedding; and fresh bedding goal on the opposite side where there was fresh clean bedding in a similar amount to home bedding. Each bedding goal was marked 6.5 cm from the centre (Figure 13, A).

Each animal was placed in the centre compartment at a 90˚ angle from the goal compartment for a fist trial and after 30 s intertrial interval the pup was again tested for a subsequent trial. Each trial was performed with the animal facing opposite sides of the equipment to balance possible animal's side turning preferences. The latency to goal was scored by the time the animal took to transpose the apparatus home bedding goal mark with both snout and forelimbs. The cut-off time of each trial was 120 s (Figure 13, B). For non-performing animals or animals that reach fresh bedding goal mark, a score of 120 s was attributed (VanRyzin et al. 2016).

Latency to goal was evaluated as an average between the two trials, resulting in one score per pup, per day.

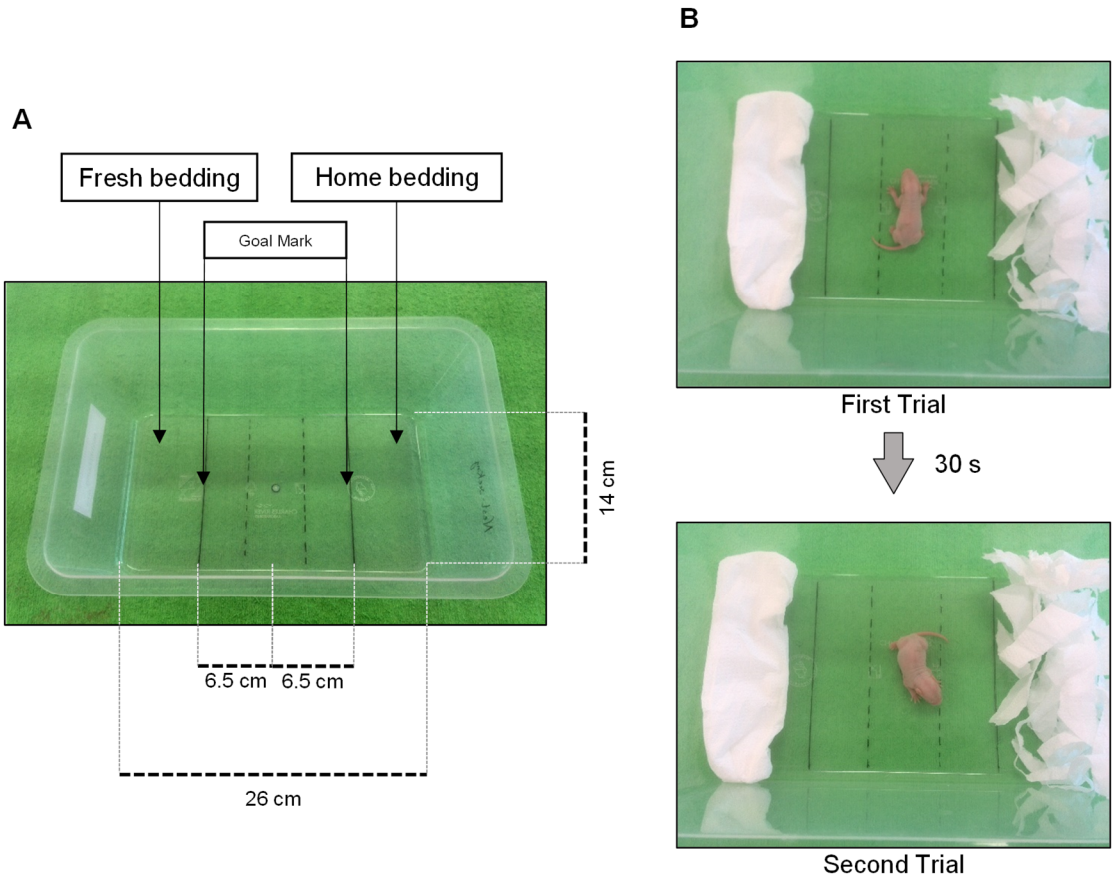


Figure 13. Representation of the nest seeking test | Rat pups PND5-PND15 ability to distinguish home bedding. **(A)** Nest seeking apparatus (26x14 cm). **(B)** Animals were evaluated on two subsequent trials for their ability to distinguishing home bedding and time was scored for home bedding goal mark crossing.

3.2.1.7. Eye opening and auditory startle

Eye opening was monitored by observation of pups PND12-PND17 and the percentage of pups with eye opened, per day, was calculated. Earing ability was evaluated by the auditory startle capability at PND11-PND14 (Baharnoori et al. 2012). Auditory startle test was used as a measure of auditory system development, which indicates maturation of somatosensory, vestibular and/or proprioceptive function (Kumar et al. 2017). Animals' capacity to produce full body startle response to a loud finger snap at a 10 cm distance was evaluated and percentage of animals that responded per day was calculated.

3.2.2. Late infancy behaviour task

All animals were gradually adapted to manipulation over several days prior to experimentation. One hour before behavioural experiments all animals were habituated to the experimentation room, which was a quiet room, with dimmed red light, controlled temperature and ventilation. Experiments were performed during the light phase of the light cycle between (8:00-17:00 h) and recorded using an overhead video camera. Between each animal, the testing arena was wiped clean with a 10 % ethanol solution.

3.2.2.1. Open field test (OPF)

The OPF was performed to access the locomotor behaviour of each animal and was performed at PND20. Animals were placed facing the wall in the centre of the arena and were left to explore the arena (45x45x40 cm) for 5 min (Figure 14). Post-hoc analysis was performed through the Any Maze software to evaluate the animals' locomotor pattern of exploration, average speed and distance travelled. Additionally, time and number of entries made by the animal in the centre of the arena was assessed as a suggestive measured of anxious-like behaviour (Machado et al. 2017).

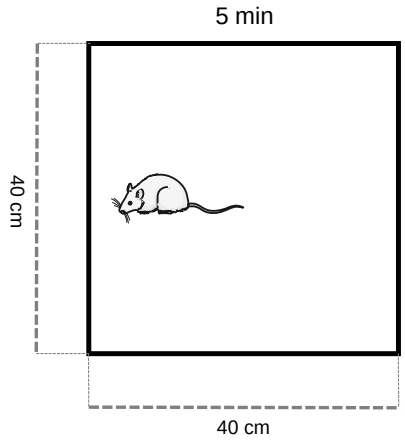


Figure 14. Schematic representation of the open field test | Locomotor behaviour was assessed on the OPF. Rats were placed on the OPF arena and were left to explore it for 5 min.

3.2.2.2. Elevated-plus maze (EPM)

The EPM test was used on PND21 animals to assess anxious-like behaviour. This test evaluates rodents' conflict between preferring protected areas, closed arm, and their innate motivation to explore new environments. Its construct validity has been confirmed by anxiogenic or anxiolytic drugs that predictably alter animals performance on the EPM (Walf & Frye 2007). For this test animals were placed facing a corner of the centre square of plus shaped platform with two open arms (45x10 cm) and two closed arms (45x10x50 cm) elevated 65 cm from the floor and were left for exploration for 5 min (Figure 15). Post-hoc analysis of animal performance in the test was achieved by "Observador" software analysis of time and number of entries on the open arm (Caetano et al. 2016).

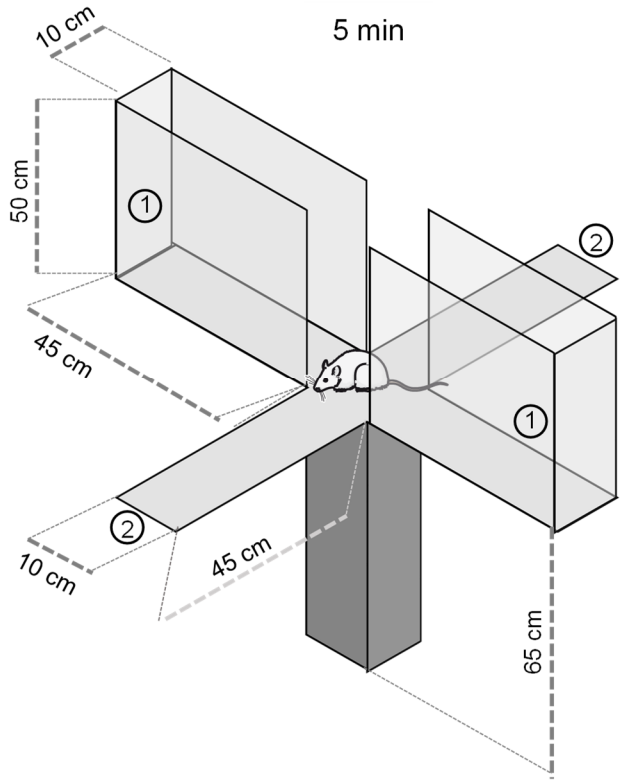


Figure 15. Schematic representation of the elevated plus maze test | Anxious-like behaviour was assessed on the EPM. Rats were placed for 5 min on EPM platform and let explore both open arms and closed arms. 1) Closed Arms; 2) Open Arms.

3.2.2.3. Novel object recognition test (NOR)

The NOR was performed at PND21 in order to access recognition memory and learning deficits. The animals were allowed to explore the arena for 5 min in the two consecutive days previous to the testing. This conditioning time was performed in order to ensure an effective familiarisation to the environment. For testing, two objects with identical colour, shape, size and texture were placed in the arena. Animals were placed facing away

from the objects and towards the centre of the opposite wall and then left to explore the arena, for a period of 5 min, and familiarise itself with two identical objects placed there. After this time animals were returned to their cages for a 2 h training-to-testing interval. When animals were again introduced to the testing arena for a 5 min novel object testing, one of the objects that was present in the familiarisation period remained, the familiar object, and the other object was substituted by a similar sized, different shape and colour object, the novel object (Figure 16). Post-hoc analysis of animal performance in the test was achieved by "Observador" software analyses of animal-object interaction. Animals that did not explored the identical objects or explored more one object than the other during familiarisation trial, were excluded from testing (Bevins & Besheer 2006).

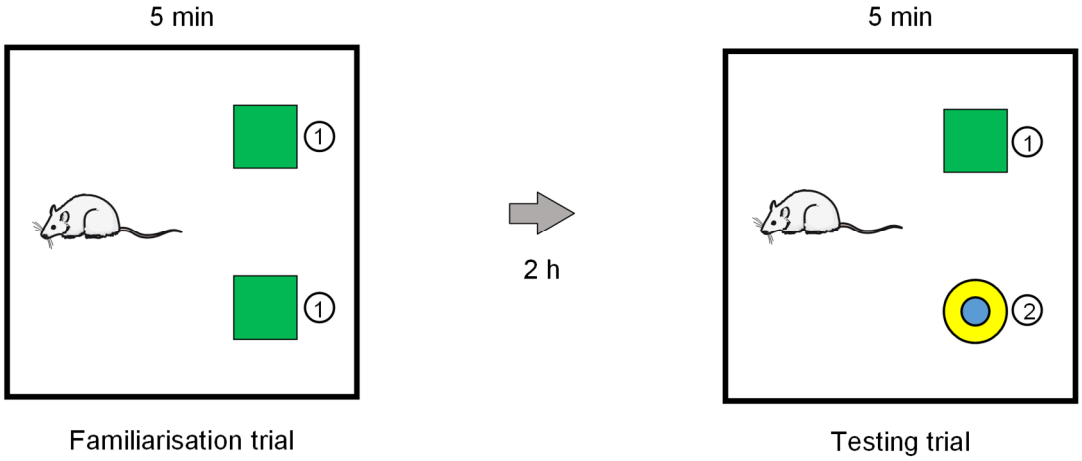


Figure 16. Schematic representation of the novel object recognition test | Learning and memory was assessed on the NOR. Rats were placed for 5 min on NOR arena with 2 identical objects (familiar objects). After 2 h rats were again placed for 5 min on the same arena where one of the objects was replaced for a novel object. 1) Familiar Object; 2) Novel Object.

The interaction was counted when animal's nose directly contacted with the objects' surface. This test evaluates animal ability to distinguish between a familiar and a novel object, being an indicator of short-term recognition memory. The animals' performance was measured through the exploration time (s) during the novel object trial, and through the recognition index:

$$Recognition\ Index = \frac{time\ spent\ in\ novel\ object}{time\ spent\ in\ familiar\ object + time\ spent\ in\ novel\ object}$$

3.3. ELISA

3.3.1. Sample collection

Blood samples collected by heart puncture after animal sacrifice were collected to an eppendorf coated with 0.5 M EDTA and centrifuged at 600 x g for 10 min at 4 ˚C, and supernant was collected and store at -80 ˚C until use.

Plasma insulin levels were measured by Mercodia Insulin ELISA kit according to manufacturer's instructions (Figure 17).

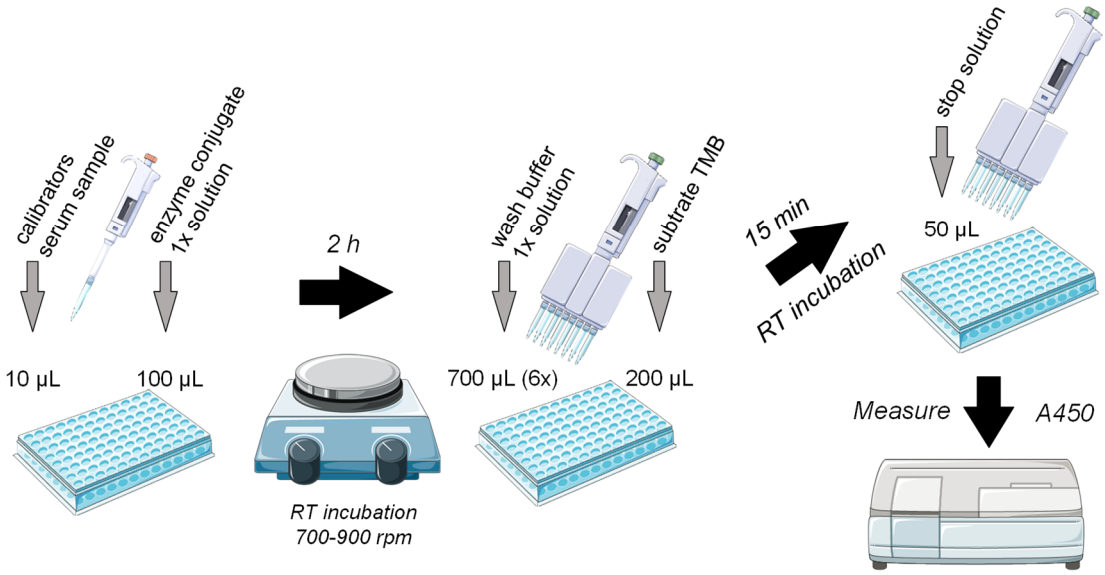


Figure 17. Mercodia Insulin ELISA kit schematic protocol | A volume of 10 µL of calibrators/samples was added to the provided coated multiwell followed by 100 µL of enzyme conjugated 1x solution. The plate was incubated at RT for 2 h under 700-900 rpm after which it was washed 6x with 700 µL of wash buffer 1x solution, followed by the addition of 200 µL of substrate TMB. After 15 min RT incubation 50 µL of stop solution was added and the plate was read at an absorbance of 450nm.

3.4. Western Blot

3.4.1. Sample collection

At PND21, female and male pups were anaesthetized with 2.5 % isoflurane (IsoFLO, Abbott Laboratories, Chicago, IL) on O_2. Under anaesthesia, animals were sacrificed by cervical dislocation. Decapitation was performed and brains were removed for hippocampal dissection. Samples were collected and frozen in liquid nitrogen and then stored at -80 ˚C.

3.4.1.1. Synaptosomal preparation

Hippocampal synaptosome extracts were prepared as previously described (Gaspar, Baptista, et al. 2010). Hippocampi was homogenized mechanically on 1mL sucrose-HEPES solution (0.32 M Sucrose; 1 mM EDTA; 10 mM HEPES; 1 mg/mL BSA; pH 7.4) in a potter by 10x up and down movement. The homogenates were centrifuged at 3,000 x g for 10 min at 4 ˚C. Supernatant was collected and centrifuged at 14,000 x g for 12 min. The pellet obtained was resuspended in 1 mL of Percoll 45 % (45 % (v/v) Percoll, 0.675 mM NaCl in KHR (Krebs-Henseleit Ring – 140 mM NaCl; 1 mM EDTA; 10 mM HEPES; 5 mM KCl; 5 mM glucose) solution pH 7.4). The resultant resuspension was centrifuged on the Eppendorf centrifuge (Eppendorf Centrifuge 5415R, Randor, PA) for 2 min at 16,000 x g at 4 ˚C. The top layer was collected and resuspended in 1 mL KHR solution for a 16,000 x g centrifugation for 2 min at 4 ˚C. Pellets were resuspended in 300 µL RIPA lysis buffer (Radioimmunoprecipitation assay buffer – 150 mM NaCl; 50 mM Tris; 5 mM EGTA; 1 % Triton X-100; 0.5 % sodium deoxycholate (DOC)) supplemented with 1 mM Na_3VO_4; 1 mM dithiothreitol (DTT), 10 mM NaF and complete miniprotease inhibitor cocktail tablet. Synaptosomal samples were them stored at -80 ˚C until the appropriate time.

3.4.1.2. Total extract preparation

Frozen hippocampi samples were dissociated on 1 mL Lysis Buffer (50 Tris-HCl, pH 7.4; 0.5 %Triton X-100) supplemented 1 mM Na_3VO_4, 1 mM DTT, 10 mM NaF and complete miniprotease inhibitor cocktail table, with a potter in a mechanic rotator. After mechanic dissociation samples were sonicated on a piston sonicator with 4 separate 2-second pulses. Samples were centrifuged for 10 min, 16,000 x g at 4 ˚C and the resultant supernatant was collected and stored at -80 ˚C until use.

3.4.2. SDS-PAGE western blot

Protein concentration was measured by colorimetric bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA). After protein concentration determination, the samples were denatured with 6x concentrated SDS sample buffer (0.5 M Tris; 30 % glycerol; 10 % SDS; 0.6 M DTT; 0.012 % Bromophenol Blue) at 95 ˚C for 5 min.

Equal protein amounts were loaded into each gel depending on the proteins of interest, as specified in Table 1. Proteins were resolved on 6-8 % SDS-Polyacrylamide gels (6 % or 8 % bis-acrylamide, trizma-HCl (1.5 M, pH 8.8), 10 %SDS, 10 % ammonium persulfate (APS), 1 % tetramethylethylenediamine (TEMED)) with a 4 % stacking gel (4 % bis-acrylamide,

trizma-HCl (0.5 M, pH 6.8), 10 % SDS, 10 % APS, 1 % TEMED). For protein separation, a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run in bicine-buffered solution (25 mM trizma; 25 mM bicine; 0.1 % SDS, pH 8.3), first at 60 V for 15 min and then at 120 V for 60 min. Proteins were transferred electrophoretically from the gel to methanol activated polyvinylidene difluoride (PVDF) membrane at 750 mA for 90 min using CAPS solution (*N*-cyclohexyl-3-aminopropanesulfonic acid) buffered solution with methanol (10 mM CAPS; 10 % methanol, pH 11.0). The membranes were blocked for 1 h with a 5 % milk solution in Tris-buffered saline (10 mM Tris; 150 mM NaCl) with 0.1 % Tween-20 (TBS-T) at room temperature (RT) and incubated overnight at 4 ˚C with primary antibody against the protein of interest (Table 1) diluted in 1 % milk in TBS-T. After 3x15min washes in TBS-T membranes were incubated with appropriate secondary antibodies (Table 1) coupled to alkaline phosphatase (AP) at RT for 1 h. After washing with TBS-T, membranes were processed for protein detection using Enhanced chemifluorescence system (ECF) on the Typhoon FLA 9000 (GE Healthcare).

Table 1 – Primary antibodies used for western blot.

Antibody	***Type***	***Host***	***Supplier***	***Protein (µg)***		***Dilution***	***Function***
				Total Extracts	***Synaptosome***		
GluA1	Polyclonal	Rabbit	Millipore AB1504	40	40	1:10,000	Glutamate Receptor; AMPA subunit
GluA2	Polyclonal	Rabbit	Millipore AB1768-I	10	10	1:500	Glutamate Receptor; AMPA subunit
GluA4	Polyclonal	Rabbit	Millipore AB1508	40	40	1:500	Glutamate Receptor; AMPA subunit
KIF1A	Monoclonal	Mouse	Bioscience 612094	40	40	1:1,000	Neuronal Motor Protein Marker
PSD-95	Monoclonal	Rabbit	Cell Signalling D27E11	40	10	1:5,000	Post-Synaptic terminal Marker
Synapsin-1	Monoclonal	Mouse	Synaptic Systems Cat. No. 106001	10	10	1:40,000	Presynaptic vesicle Marker
Synaptophysin	Monoclonal	Mouse	Sigma S5768	10	10	1:40,000	Presynaptic vesicle Marker
VGluT-1	Monoclonal	Mouse	Santa Cruz Sc-377425	40	40	1:100	Glutamatergic Synapse Marker
GAPDH	Polyclonal	Goat	Alfagene AB0049-200	-	-	1:5000	Loading control

Membranes were then reincubated for loading control protein, (glyceraldehyde 3-phosphate dehydrogenase - GAPDH) (Table 1) primary antibody diluted in 1 % milk in TBS-T, overnight at 4 ˚C. After 3x15 min washes in TBS-T, membranes were incubated with

secondary antibody (Table 2) coupled to horseradish peroxidase (HRP) at RT for 1 h. After 3x15 min washes with TBS-T, membranes were processed for protein detection using a commercial enhanced chemiluminescence (ECL) detection method kit on the ImageQuantTM LAS 500 (GE Healthcare). Subsequently, membranes' intensity was quantified through the ImageQuant 5.0 software (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

Table 2 –Secondary antibodies used for western blot.

Antibody	*Detection Method*	*Host*	*Supplier*	*Dilution*
AP Goat Anti-Mouse IgG	ECF	Goat	Sigma A3562	1:10,000
AP Goat Anti-Rabbit IgG	ECF	Goat	Sigma A3687	1:10,000
HRP Rabbit Anti-Goat	ECL	Rabbit	Alfagene (Thermo Fisher Scientific) LTI 611620	1:10,000

3.5. Cresyl Violet

At PND21, female and male pups from CTRL and STZ dams were anaesthetized with intraperitoneal injection of 90 mg/Kg Ketamine (Nimatek) and 10 mg/Kg Xylazine (Ronpum 2%), for transcardiac perfusion. To determine the depth of anaesthesia the toe pinch-response method was performed and only upon a surgical plane of anaesthesia level cardiac perfusion procedure was initiated. Animals were immobilised on a surgical tray, the heart was exposed and penetrated through the posterior left ventricle by a needle connected to a peristaltic pump for perfusion. Perfusion with PBS 1x (Phosphate Buffered Saline – 137 mM NaCl; 2.7 mM KCl; 1.8 mM KH_2PO_4; 10 mM $Na_2HPO_4 \cdot 2H_2O$; pH 7.4) solution was firstly used for blood removal from the vasculature followed by 4 % paraformaldehyde (PFA) solution (in PBS 1x, pH 7.4) for fixation. Brains were removed and left in 4 % PFA solution overnight, and then transferred to a 30 % sucrose solution in PBS 1x till the tissue density made the brain sink. Sample brains were cryopreserved on Criomatrix embedding resin for a 30 µm thickness sectioning on Cryostat (LEICA CM3050 S, Germany) at -20 ˚C chamber temperature and -19 ˚C object temperature. Slices were transferred to a 24 multiwell plate filled with cryoprotection solution (30 % Sucrose; 30 % Ethylene glycol; 10 mM phosphate buffer, pH 7.2) for storage at 4 ˚C.

Cryosectioned slices from the dorsal hippocampal region, located at the stereotaxic coordinates of interaural 6.08 mm and bregma 2.92 mm (Paxinos & Charles 2007) (Figure 18, A; B) were mounted onto gelatinized slides and were left to air dry at RT. Slides were immersed in Xylene for 5 min followed by a 3 min dip on 95 % ethanol, 3 min dip on 70 %

ethanol and 3 min dip on Distilled water. Then slices were immersed on pre-heated cresyl violet for 8 min at 60 ˚C. Slides were dipped in distilled water for washing for 3 min, followed by dipping on a sequential increasing percentage of ethanol, first 70 % for 3 min, then 95 % for 1 min and 100 % for a few seconds. Finally, they were immersed on xylene for 5 min and left to air dry. Slides were mounted with DPX mounting medium, left to dry and stored at RT until imaging. Images from the cornu ammonis (CA)1, CA3 and dentate gyrus (DG) hippocampal subregions were acquired on a light microscope (Leica DM 4000 B) under 5x, 10x and 40x magnification objective.

For image analysis, 40x magnification images from each hippocampal subregion- were selected. For each image 3 independent measures of the layers thickness (in μm) were taken using the ImageJ software (Figure 18; C-H).

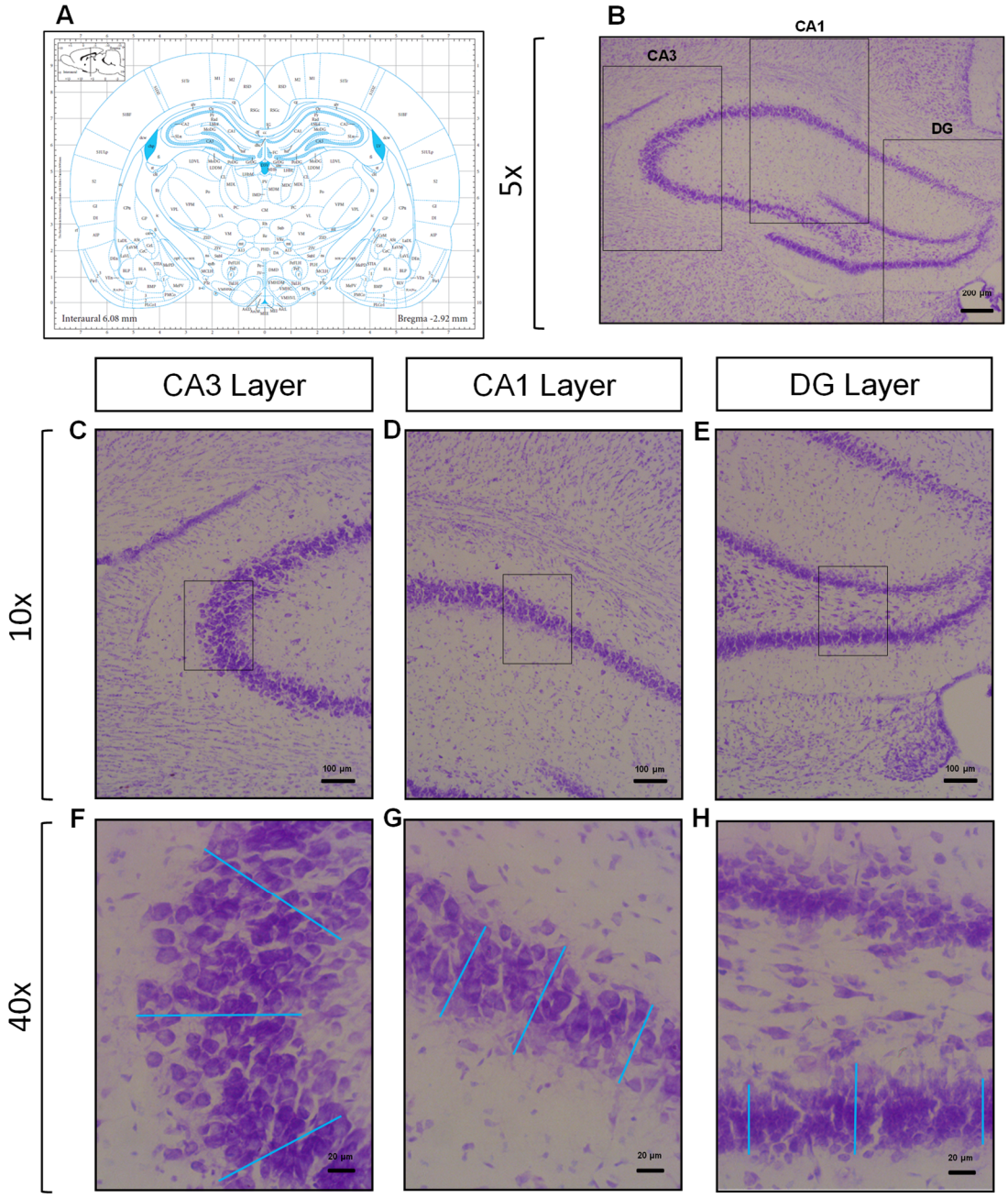


Figure 18. Representation of cresyl violet staining thickness measurements of the selected hippocampal CA3, CA1, DG subregions | Rat dorsal hippocampal slices stained with cresyl violet. **(A)** Hippocampal slices were selected from the dorsal hippocampal region, located at the stereotaxic coordinates of interaural 6.08 mm and bregma 2.92 mm (Paxinos & Charles 2007) **(B)** Image composition of hippocampus acquired on the brighfield microscope at magnification of 5x; Scale bar: 200 µm. **(C-E)** 10x magnification images of CA3, CA1 and DG subregions of the hippocampus; Scale bar: 100 µm; **(F-H)** 40x magnification images of CA3, CA1 and DG subregions of the hippocampus with 3 representative measurements (blue lines) of layer thickness; Scale bar: 20 µm). Adapted from Paxinos & Charles 2007.

3.6. Statistical Analysis

All data related to offspring, with exception of NOR-related data, was analysed separately for male and female offspring.

Outliers were identified with an online outlier calculator from GraphPad Software (https://graphpad.com/quickcalcs/Grubbs1.cfm) ($\alpha = 0.05$) and excluded when found.

Statistical analysis was performed with Graph Pad Prism 6 software. Results were shown as Mean ± standard error of the mean (SEM). Differences between groups were analysed by Student's *t*-test. Differences were considered significant at $p < 0.05$.

3.7. Reagents

Table 3 – List of reagents and commercial kits used.

Reagent	*Supplier*
Acrylamide/Bis 30% Solution	Biorad 161-0158
Ammonium Persulfate (APS)	Sigma-Aldrich A3678
BCA protein assay Kit	Thermo Scientific 23225
Bicine	Acros organics 172650010
CAPS	Thermo Scientific BP321-500
cOmplete(TM), Mini Protease Inhibitor	Sigma-Aldrich 11836153001
Cresyl Violet	Sigma-Aldrich C5042
DL-Dithiothreitol (DTT)	Sigma-Aldrich D-9779
Dodecyl Sulfate Sodium Salt (SDS)	Sigma-Aldrich 436143
DPX mounting medium	Fluka 44581
ECF substract	Amersham RPN5785
Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-*tetraacetic acid* (EGTA)	Sigma-Aldrich E-4378
Ethanol	Enzymatic VR11202
Ethylene glycol	Sigma-Aldrich 293237
Ethylenediaminetetracetic acid disodium salt dihydrate (EDTA)	Sigma-Aldrich ED2SS-100G/E5134
Glucose	Sigma-Aldrich G8270
Glycerol	Sigma-Aldrich G-551616
Hydrochloric acid (HCl)	Merk/AppliChem 1.00317.2500
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Biochemical 441475K
Methanol	Sigma-Aldrich 32213
Paraformaldehyde (PFA)	Merk 8.18715.1000

cont.

cont.

Reagent	***Supplier***
Percoll 100%	Sigma-Aldrich P-1644
Polyvinylidene difluoride (PVDF) membranes	Millipore IPVH00010
Potassium Chloride (KCl)	Merck 4936
Potassium di-hydrogen phosphate (KH_2PO_4)	Panreac 131509
Sodium Chloride (NaCl)	Sigma-Aldrich S7653
Sodium Deoxycholate (DOC)	Laborspirit D6750-0010
Sodium fluoride, anhydrous (NaF)	Sigma-Aldrich 450022
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich S6508
Streptozotocin	Sigma-Aldrich S-0130
Sucrose	Fisher S/8600/60
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich T9281
Tris(hydroxymethyl)aminomethane (Trizma)	Merck 1.08386.1000
Tris(hydroxymethyl)aminomethane hydrochloride (Trizma-HCl)	Merck 1.08219.0750
TRITON X100	Sigma-Aldrich X100
TWEEN-20	Merck 8.22184.0500
WesternBright Sirius HRP substrate	GRISP K-12043-D20
Xylene	Panreac 131769

Chapter 4

4. Results

4. Results

4.1. Animals' Metabolic Characterisation

After mating, weight of both diabetic and control female dams were regularly monitored. At GD21, CTRL dams weight did not significantly differ comparing with STZ-injected dams (Figure 19, A.). After birth, dams glycaemia was measured to confirm normoglycemia in control females and hyperglycaemia (<250 mg/dL) in STZ-injected ones. Diabetic females presents a statistically significant increase in glycaemia relatively to control dams (GD21 – CTRL: 89.1 ± 4.8 mg/dL; STZ: 459.3 ± 27 mg/dL; $p < 0.0001$) (Figure 19, B).

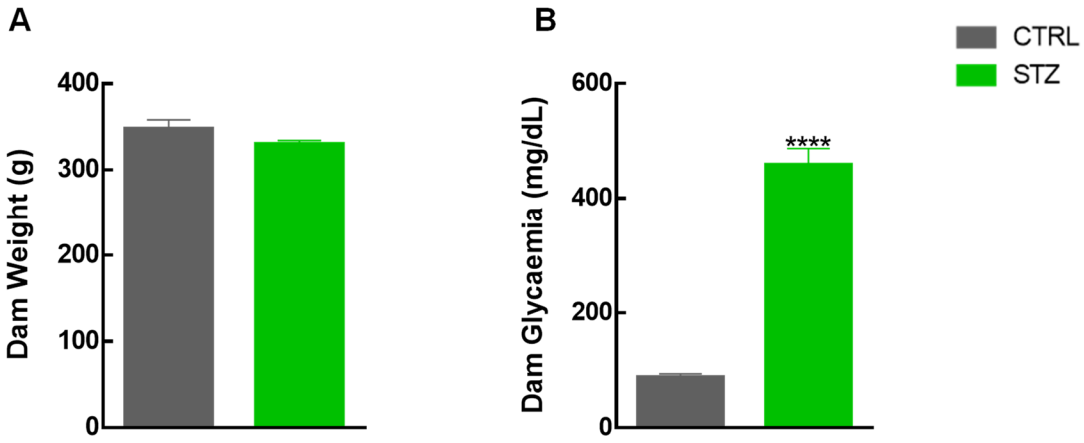


Figure 19. Maternal bodyweight and glycaemia at the end of gestation | Results of diabetic (STZ) (green) and control (CTRL) (grey) dams weight (A) and glycaemia (B) are presented as the mean ± SEM. **(A)** Dams were weighted at GD21 (n=8-10). **(B)** Dams' glycaemia was measured at GD21 (n=9-10). Statistical analysis was assessed with Student's *t*-test, ****p < 0.0001, compared with control group.

4.1.1. Effect of maternal diabetes on offspring's bodyweight, glycaemia and plasma insulin levels

Henceforth, the offspring of diabetic dams will be referred as STZ even though these animals were not exposed to STZ at any point, only their mothers. The same way, offspring of non-diabetic females will be referred as CTRL.

It is described that foetal exposure to a hyperglycaemic state in womb alters maternal diabetic offspring's metabolism with consequences that persist later in life and particularly interfere with weight and glucose/insulin metabolism (L Aerts et al. 1990).

Therefore, after birth, we weighed the offspring at PND0, PND7, PND14 and PND21 to follow neonates' weight (g) gain (Figure 20, A; B). In males, no differences were observed at PND0. However, at PND7 there was a tendency for lower weight in STZ compared to

CTRL offspring (PND7 – CTRL♂: 13.6 ± 0.3 g; STZ♂: 12.3 ± 0.6 g; p = 0.081), that at PND14 and PND21 became statistically significant with lower weight in STZ compared to CTRL offspring (PND14 – CTRL♂: 25.8 ± 0.6 g; STZ♂: 20.8 ± 1.4 g; p < 0.01 | PND21 – CTRL♂: 39.7 ± 0.9 g; STZ♂: 30.1 ± 2.2 g; p < 0.001). In female offspring similar results

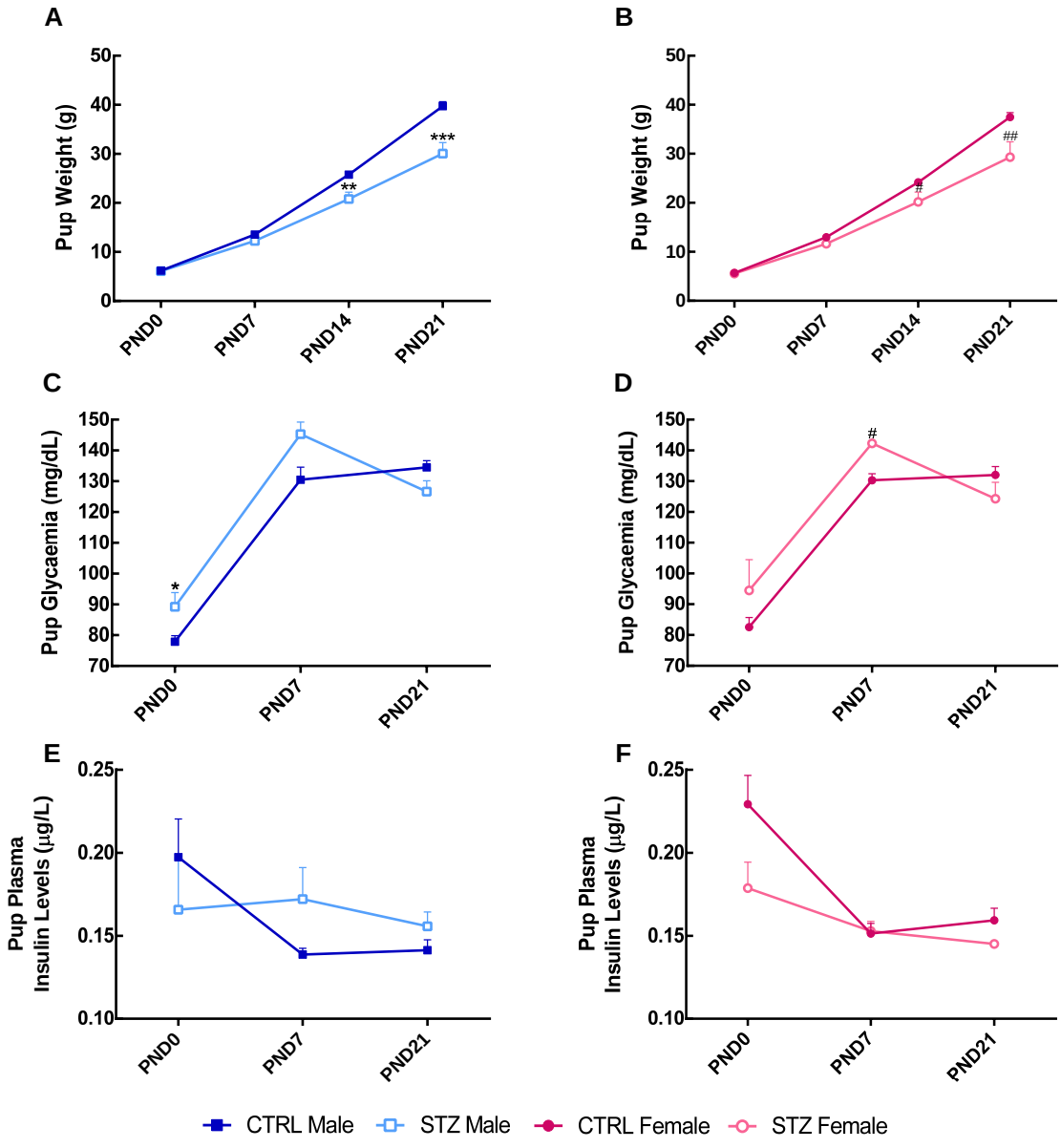


Figure 20. Effect of maternal diabetes in male and female offspring's bodyweight, glycaemia and plasma insulin levels | Results regarding diabetic dams´ offspring (STZ) and control (CTRL) ones are presented as the mean ± SEM. Left panel shows results for male offspring (blue) and the right panel shows results for female offspring (pink). **(A, B)** Pups were weight at PND0, 7, 14 and 21. Pups from 5 independent litters from both control and diabetic dams (n=13-27) were used. **(C, D)** Pup's glycaemia was measured at PND0, 7 and 21 Pups from 3 independent litters from both control and diabetic dams were used at PND0 (n=6-15); 3 independent litters from control and 1 from diabetic dam at PND7 (n=3-15) and 5 separate litters from both control and diabetic dams at PND21 (n=12-27). **(E, F)** Pup's circulating insulin levels were measured by ELISA assay in plasma fractions isolated from blood at PND0, 7 and 21, representing the results from 2 separate litters from both control and diabetic dams at PND0 (n=3-4); 1 litter from control and diabetic dam at PND7 (n=3-4) and 3 separate litters from both control and diabetic dams at PND21 (n=3). Statistical analysis was assessed with Student's *t*-test for each time point; *p < 0.05, **p < 0.01, ***p < 0.001 compared with male control group; #p < 0.05, ##p < 0.01, compared with female control group.

were observed. There were no significant differences at both PND0 and PND7 but at PND14 and PND21 STZ offspring's weight was significantly lower compared to CTRL offspring (PND14 – CTRL♀: 24.2 ± 0.7 g; STZ♀: 20.2 ± 2.0 g; $p < 0.05$ | PND21 – CTRL♀: 39.7 ± 0.9 g; STZ♀: 30.1 ± 2.2 g; $p < 0.01$). All values for pups' weight are summarised in Supplementary Data (Table 4).

We measured glycaemia (mg/dL) in the offspring at PND0, PND7, and PND21 to evaluate circulating basal glucose levels (Figure 20, C; D). In males, at PND0, STZ offspring had significantly higher glycaemia relative to CTRL offspring (PND0 – CTRL♂: 77.9 ± 1.9 mg/dL; STZ♂: 89.2 ± 4.6 mg/dL; $p < 0.05$), however, at PND7 only a tendency was observed (PND7 – CTRL♂: 130.5 ± 4.0 mg/dL; STZ♂: 145.3 ± 3.9 mg/dL; $p = 0.085$). At PND21, a strong tendency was observed for STZ to have lower glycaemia comparing with CTRL offspring (PND21 – CTRL♂: 134.5 ± 2.2 mg/dL; STZ♂: 126.6 ± 3.6 mg/dL; $p = 0.060$). In females, we observed that at PND0 STZ offspring did not had statistically significant differences in glycaemia comparing with CTRL group. However, at PND7 it was observed that STZ had significantly higher glycaemia relative to CTRL (PND7 – CTRL♀: 130.3 ± 2.1 mg/dL; STZ♀: 142.3 ± 0.3 mg/dL; $p < 0.05$). At PND21, no statistical differences were observed, but as for males a tendency to lower glycaemia value observed comparing with CTRL. All values for pup glycaemia are summarised in Supplementary Data (Table 5).

At PND0, PND7, and PND21 in order to evaluate circulating insulin levels we isolated plasma from CTRL and STZ offspring blood samples and performed an ELISA to quantify insulin levels (µg/L) (Figure 20, E; F). For males, we did not observe any significant differences at PND0, PND7 and PND21. For females, at PND0, STZ offspring had a tendency for a lower insulin level relative to control (PND0 – CTRL♀: 0.159 ± 0.007 µg/L; STZ♀: 0.145 ± 0.002 µg/L; $p = 0.073$), however, at PND7 and PND21 this tendency was not observed. All values for pup glycaemia are summarised in Supplementary Data (Table 6).

4.2. Developmental Behavioural Testing

Since maternal diabetes is described to influence neurodevelopment (DeRegnier et al. 2000; Nelson et al. 2000; ter Braak et al. 2002) and may also induce psychomotor skill deficits (Ornoy et al. 1998; Rizzo et al. 1995) in the offspring of diabetic mothers, we decided to perform a battery of test to evaluate reflex development, the achievement of physical development milestones and locomotion throughout an early life phase.

4.2.1. Maternal diabetes induces a delay in male and female offspring's surface righting reflex, negative geotaxis reaction, as well as, cliff aversion behaviour

Reflex development was evaluated in pups at PND5-PND10. Animals were placed on their back and the time (s) of righting was noted (Figure 21, A; B). For males, at PND5 and PND6 a tendency could be observed for STZ offspring to take more time to right themselves relatively to CTRL (PND5 – CTRL♂: 1.2 ± 0.1 s; STZ♂: 1.4 ± 0.1 s; $p = 0.078$ | PND6 – CTRL♂: 1.0 ± 0.0 s; STZ♂: 1.1 ± 0.1 s; $p = 0.059$), at PND8 the time of STZ animals to right themselves is significantly higher than CTRL offspring (PND8 – CTRL♂: 1.0 ± 0.0 s; STZ♂: 1.4 ± 0.1 s; $p < 0.01$). At PND7, PND9 and PND10 no statistically significant differences were observed. Likewise we also observed at PND5 and PND6 a tendency for STZ female offspring to take more time to right themselves relatively to CTRL (PND5 – CTRL♀: 1.1 ± 0.1 s; STZ♀: 1.5 ± 0.2 s; $p = 0.053$ | PND6 – CTRL♀: 1.0 ± 0.0 s; STZ♀: 1.1 ± 0.1 s; $p = 0.096$). At PND8 STZ the time of STZ animals to right themselves is significantly higher than CTRL offspring (PND8 – CTRL♀: 1.0 ± 0.0 s; STZ♀: 1.5 ± 0.2 s; $p < 0.01$). At PND7, PND9 and PND10 there were no significant differences. All values for pup righting reflex time are summarised in Supplementary Data (Table 7).

Negative geotaxis reaction was evaluated in pups at PND5-PND14. Animals were placed face-down and tested for latency (s) to reverse orientation and face upwards on a 35-degree inclined platform for negative geotaxis reaction was evaluated (Figure 21, C; D). For male offspring, at PND5-PND7 and PND12 no significant differences were observed but for PND8-PND11, PND13 and PND14 STZ offspring showed significantly slower reaction than CTRL offspring (PND8 – CTRL♂: 14.3 ± 1.7 s; STZ♂: 19.3 ± 1.8 s; $p < 0.05$ | PND9 – CTRL♂: 7.5 ± 0.8 s; STZ♂: 12.6 ± 1.0 s; $p < 0.01$ | PND10 – CTRL♂: 8.1 ± 0.9 s; STZ♂: 12.6 ± 1.0 s; $p < 0.05$ | PND11 – CTRL♂: 7.8 ± 0.7 s; STZ♂: 12.5 ± 1.5 s; $p < 0.01$ | PND13 – CTRL♂: 6.9 ± 0.6 s; STZ♂: 12.1 ± 1.3 s; $p < 0.001$ | PND14 – CTRL♂: 6.5 ± 0.5 s; STZ♂: 10.4 ± 1.5 s; $p < 0.05$). For female offspring at PND5-PND7 and PND10-PND12 no statistical differences were observed. However, at PND8 and PND9 we could observe a tendency for slower reaction in STZ offspring related to control (PND8 – CTRL♀: 17.5 ± 2.2 s; STZ♀: 23.7 ± 2.5 s; $p = 0.075$ | PND9 – CTRL♀: 8.9 ± 1.3 s; STZ♀: 13.7 ± 2.3 s; $p = 0.097$). Only at PND13 and PND14 STZ female offspring had slower reaction comparing with CTRL animals (PND13 – CTRL♀: 5.8 ± 1.5 s; STZ♀: 13.5 ± 2.3 s; $p < 0.001$ | PND14 – CTRL♀: 6.6 ± 0.6 s; STZ♀: 12.0 ± 2.5 s; $p < 0.05$). All values for pup negative geotaxis reaction time are summarised in Supplementary Data (Table 8).

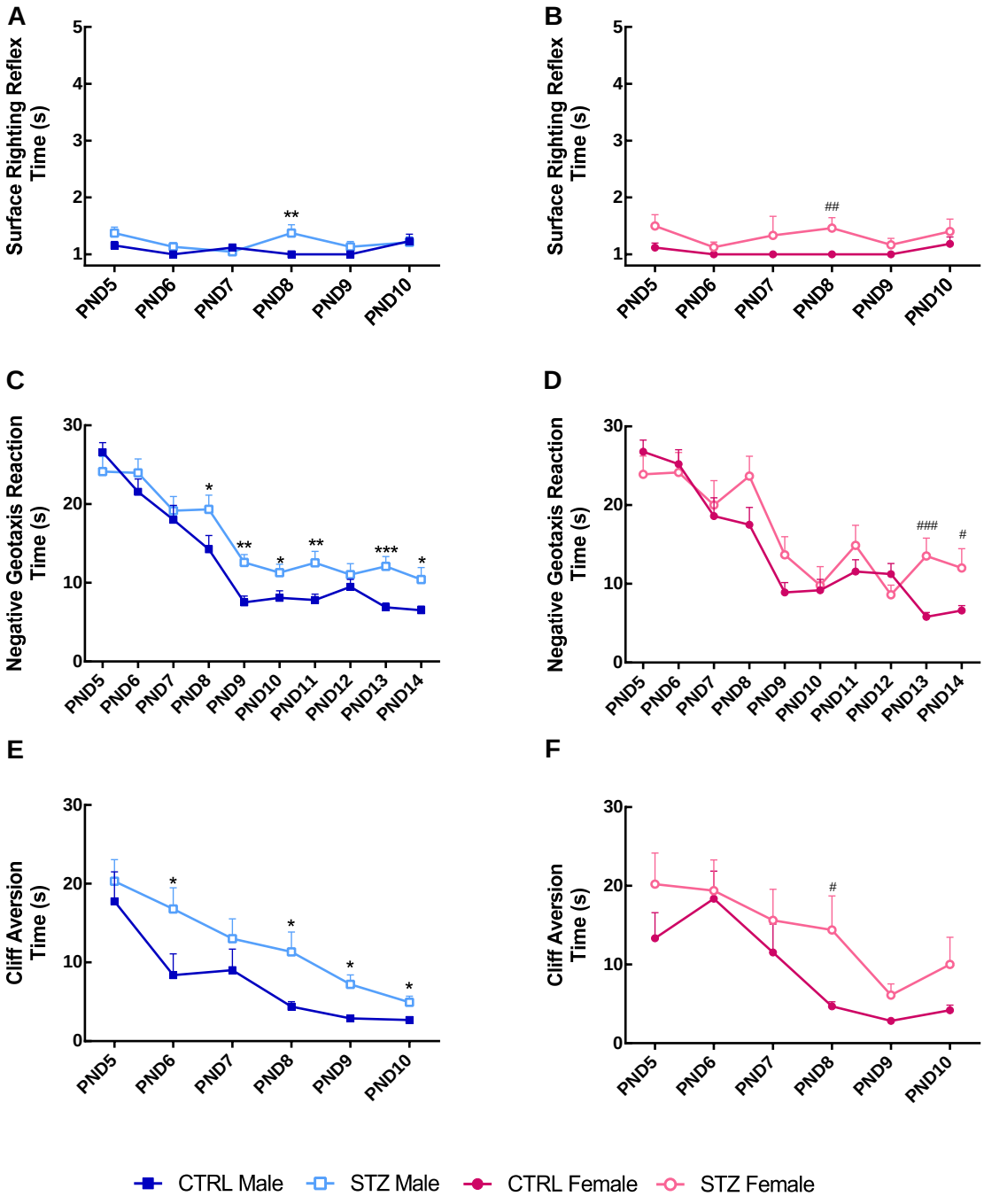


Figure 21. **Maternal diabetes induces a delay in male and female offspring's surface righting reflex, negative geotaxis reaction, as well as, cliff aversion behaviour |** Results regarding diabetic dams´ offspring (STZ) and control (CTRL) ones are presented as the mean ± SEM. Left panel shows results for male offspring (blue) and the right panel shows results for female offspring (pink). **(A, B)** Rat pups were tested for the time to successfully right themselves (surface righting reflex) (s) from PND5-PND10. Pups from 5 independent litters from both control and diabetic dams (n=10-26) were used. **(C, D)** Rat pups were tested for the latency (s) to reverse orientation and face upwards on an incline plane, for from PND5-PND14. Pups from 5 independent litters from both control and diabetic dams (n=10-27) were used. **(E, F)** Rat pups were tested for time (s) to retract from an edge, from PND5-PND10. Pups from 3 independent litters from control and 4 from diabetic Dams (n=6-19) were used. Statistical analysis was assessed with Student's *t*-test for each time point; *p < 0.05, **p < 0.01, ***p < 0.001 compared with male control group; #p < 0.05, ##p < 0.01, ###p < 0.001 compared with female control group.

Finally, we performed the cliff aversion test in pups at PND5-PND10. Animals were placed on an elevated flat surface hanging over the edge and time (s) of retraction from the edge was counted (Figure 21, E; F). For male offspring, at PND5 and PND7 no significant differences were observed but at PND6, PND8-PND10 STZ offspring took longer than CTRL to avoid the edge (PND6 – CTRL♂: 8.4 ± 2.7 s; STZ♂: 16.8 ± 2.7 s; $p < 0.05$ | PND8 – CTRL♂: 4.3 ± 0.6 s; STZ♂: 11.3 ± 2.5 s; $p < 0.05$ | PND9 – CTRL♂: 2.9 ± 0.2 s; STZ♂: 7.2 ± 1.2 s; $p < 0.05$ | PND10 – CTRL♂: 2.7 ± 0.2 s; STZ♂: 4.9 ± 0.8 s; $p < 0.05$). For female offspring, at PND5-PND7 and PND10 no statistical differences were observed, at PND8 we could observe that STZ offspring took significantly longer than CTRL to retract from the edge (PND8 – CTRL♀: 4.7 ± 0.6 s; STZ♀: 14.4 ± 4.3 s; $p < 0.05$) and at PND9 STZ females showed a tendency to take more time relative to CTRL (PND9 – CTRL♀: 2.8 ± 0.3 s; STZ♀: 6.1 ± 1.4 s; $p = 0.090$). All values regarding time pups took to retract from the edge are summarised in Supplementary Data (Table 9).

4.2.2. Maternal diabetes induces neuromuscular strength and locomotor impairments in male and female offspring

Pups were tested for their strength in the wire suspension test. The time (s) pups PND10-PND14 spent hanging on the wire was scored until they fell to the padded drop zone (Figure 22, A; B). For males, at PND10-PND13 no statistically significant differences were observed, but at PND14 STZ offspring hanged less time on the wire than CTRL ones (PND14 – CTRL♂: 9.8 ± 0.2 s; STZ♂: 6.8 ± 0.7 s; $p < 0.001$). Regarding female pups, at PND10-PND12 and PND14, we observed no statistical differences, but at PND13 STZ offspring hanged less time on the wire than CTRL pups (PND13 – CTRL♀: 8.8 ± 0.5 s; STZ♀: 6.5 ± 1.0 s; $p < 0.05$). All values related to pup wire hung suspension time are summarised in Supplementary Data (Table 10).

Locomotion was assessed at PND5-PND14 by placing the pup in the centre of a 13 cm diameter circle and by recording the time (s) for the animal to fully exit the arena (Figure 22. C; D). For male offspring at PND5, PND7, PND10 and PND14 no significant differences were observed, at PND8 and PND11 there was a strong tendency for STZ offspring taking longer time to exit the arena than CTRL ones (PND8 – CTRL♂: 25.4 ± 1.2 s; STZ♂: 28.4 ± 0.9 s; $p = 0.072$ | PND11 – CTRL♂: 22.9 ± 1.3 s; STZ♂: 26.9 ± 1.4 s; $p = 0.051$), at PND6, PND9, PND12 and PND13 STZ offspring showed statistical significant slower locomotion that CTRL offspring (PND6 – CTRL♂: 26.8 ± 1.2 s; STZ♂: 30.0 ± 0.0 s; $p < 0.05$ | PND9 – CTRL♂: 24.9 ± 2.1 s; STZ♂: 29.7 ± 0.2 s; $p < 0.05$ | PND12 – CTRL♂: 19.3 ± 1.5 s; STZ♂: 25.2 ± 1.3 s; $p < 0.01$ | PND13 – CTRL♂: 16.9 ± 1.5 s; STZ♂: 24.3 ± 1.7 s; $p < 0.01$). For female offspring, at PND5-PND8 and PND10 no statistical differences were observed, at

PND9 and PND11-PND14 we could observe a significant slower locomotion in STZ offspring comparing with CTRL (PND9 – CTRL♀: 22.0 ± 2.8 s; STZ♀: 29.0 ± 0.7 s; $p < 0.05$ | PND11 – CTRL♀: 19.3 ± 1.5 s; STZ♀: 28.0 ± 1.0 s; $p < 0.001$ | PND12 – CTRL♀: 19.8 ± 1.9 s; STZ♀: 26.5 ± 1.6 s; $p < 0.05$ | PND13 – CTRL♀: 16.5 ± 2.3 s; STZ♀: 28.2 ± 1.2 s; $p < 0.001$ | PND14 – CTRL♀: 12.1 ± 2.0 s; STZ♀:19.4 ± 2.5 s; $p < 0.05$). All values concerning the latency for rat offspring to leave the arena are summarised in Supplementary Data (Table 11).

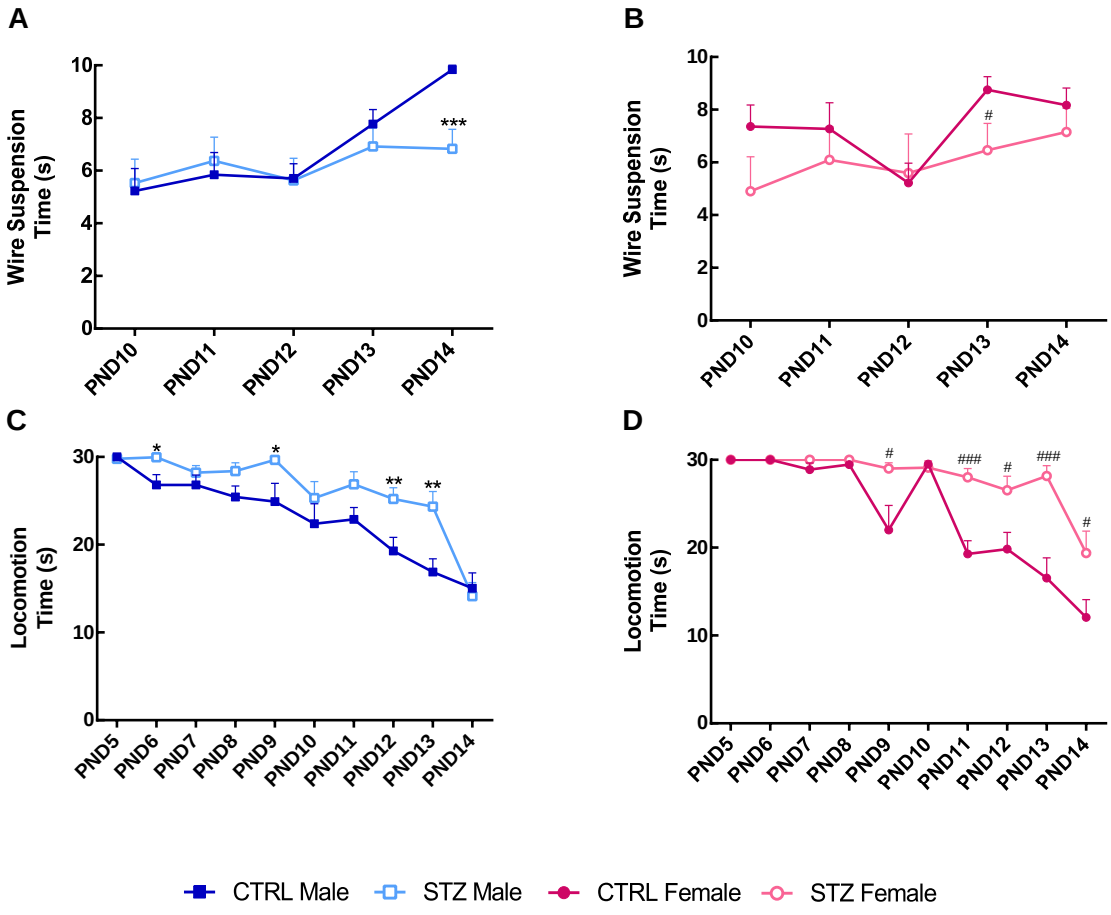


Figure 22. Maternal diabetes induces neuromuscular strength and locomotor impairments in male and female offspring | Results regarding diabetic dams´ offspring (STZ) and control (CTRL) ones are presented as the mean ± SEM. Left panel shows results for male offspring (blue) and the right panel shows results for female offspring (pink). **(A, B)** Time (s) rat pups hung from their forepaws before falling, from PND10-PND14. Pups from 5 independent litters from both control and diabetic dams (n=10-27) were used. **(C, D)** Latency (s) for rat pups to leave the 13 cm circle area, from PND5-PND14. Pups from 5 independent litters from both control and diabetic dams (n=9-27) were used. Statistical analysis was assessed with Student's *t*-test for each time point; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with male control group; #$p < 0.05$, ###$p < 0.001$ compared with female control group.

4.2.3. Maternal diabetes impairs male and female offspring's nest seeking behaviour

Pups (PND5-PND15) were tested for their latency (s) to goal (home-bedding) on the nest seeking test. This test evaluates locomotion, as well as the ability to identify and locate the maternal nest that depends on display of adequate olfactory, motor and discriminatory ability to discern nest bedding from home bedding (Baharnoori et al. 2012) (Figure 23, A; B). For male offspring, at PND5, PND7-PND13 no significant differences were observed. Nevertheless, at PND6, PND14 and PND15 STZ offspring showed statistically significant higher latency to goal than CTRL offspring (PND6 – CTRL♂: 50.0 ± 10.3 s; STZ♂: 83.8 ± 9.3 s; $p < 0.05$ | PND14 – CTRL♂: 2.7 ± 0.2 s; STZ♂: 5.9 ± 0.8 s; $p < 0.001$ | PND15 – CTRL♂: 2.4 ± 0.2 s; STZ♂: 12.8 ± 4.0 s; $p < 0.01$). In which concerns to female offspring, at PND5-PND7, PND9-PND11 and PND14 no significant differences were observed. Still, at PND8 we could observe a tendency for STZ offspring to have higher latency to goal than CTRL (PND8 – CTRL♀: 23.9 ± 5.5 s; STZ♀: 52.0 ± 13.9 s; $p = 0.078$), whereas at PND12 and PND15 STZ offspring had significantly higher latency to goal than CTRL (PND12 – CTRL♀: 4.5 ± 0.5 s; STZ♀: 23.5 ± 7.7 s; $p < 0.01$ | PND15 – CTRL♀: 2.9 ± 0.2 s; STZ♀: 8.4 ± 1.6 s; $p < 0.001$). Nevertheless, at PND13 STZ offspring had lower latency to goal than CTRL (PND13 – CTRL♀: 22.2 ± 6.5 s; STZ♀: 5.9 ± 0.8 s; $p < 0.05$). All values for pup nest seeking time are summarised in Supplementary Data (Table 12).

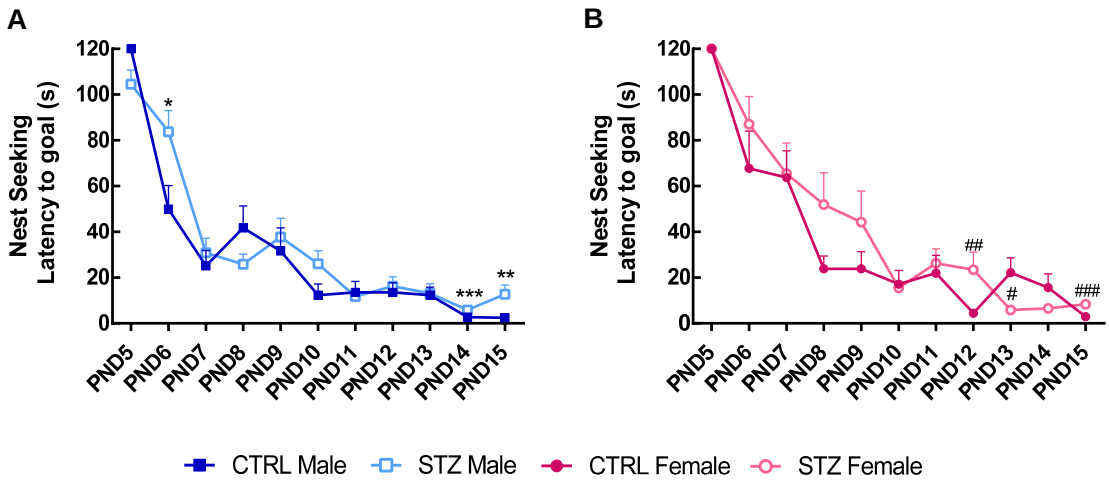


Figure 23. Maternal diabetes impairs male and female offspring's nest seeking behaviour | Results regarding diabetic dams´ offspring (STZ) and control (CTRL) ones are presented as the mean ± SEM. Left panel shows results for male offspring (blue) and the right panel shows results for female offspring (pink). **(A, B)** Pups were tested for their latency to achieve goal (home-bedding) on the nest seeking test (s) from PND5-PND15. Pups from 5 independent litters from both control and diabetic dams (n=5-27) were used. Statistical analysis was assessed with Student's *t*-test for each time point; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with male control group; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, compared with female control group.

4.2.4. Maternal diabetes induces a delay in male and female offspring's eye opening as well as in the achievement of auditory startle response

Pups were evaluated for the achievement of physical developmental milestones. Pups (PND12-PND17) were observed for the day of eye opening and percentage of eyes open, per day, was obtained (Figure 24, A; B). For males at PND12-PND14 no significant differences were observed but from PND15-PND17 STZ offspring had a significantly delay in eye opening compared to CTRL (PND15 – CTRL♂: 59.3 ± 9.6 %; STZ♂: 25.0 ± 9.0 %; $p < 0.01$ | PND16 – CTRL♂: 100.0 ± 0.0 %; STZ♂: 36.8 ± 11.4 %; $p < 0.0001$ | PND17 – CTRL♂: 100.0 ± 0.0 %; STZ♂: 62.5 ± 10.1 %; $p < 0.001$). For females, at PND12-PND14 we did not observe statistical differences, whereas at PND15 STZ offspring had a

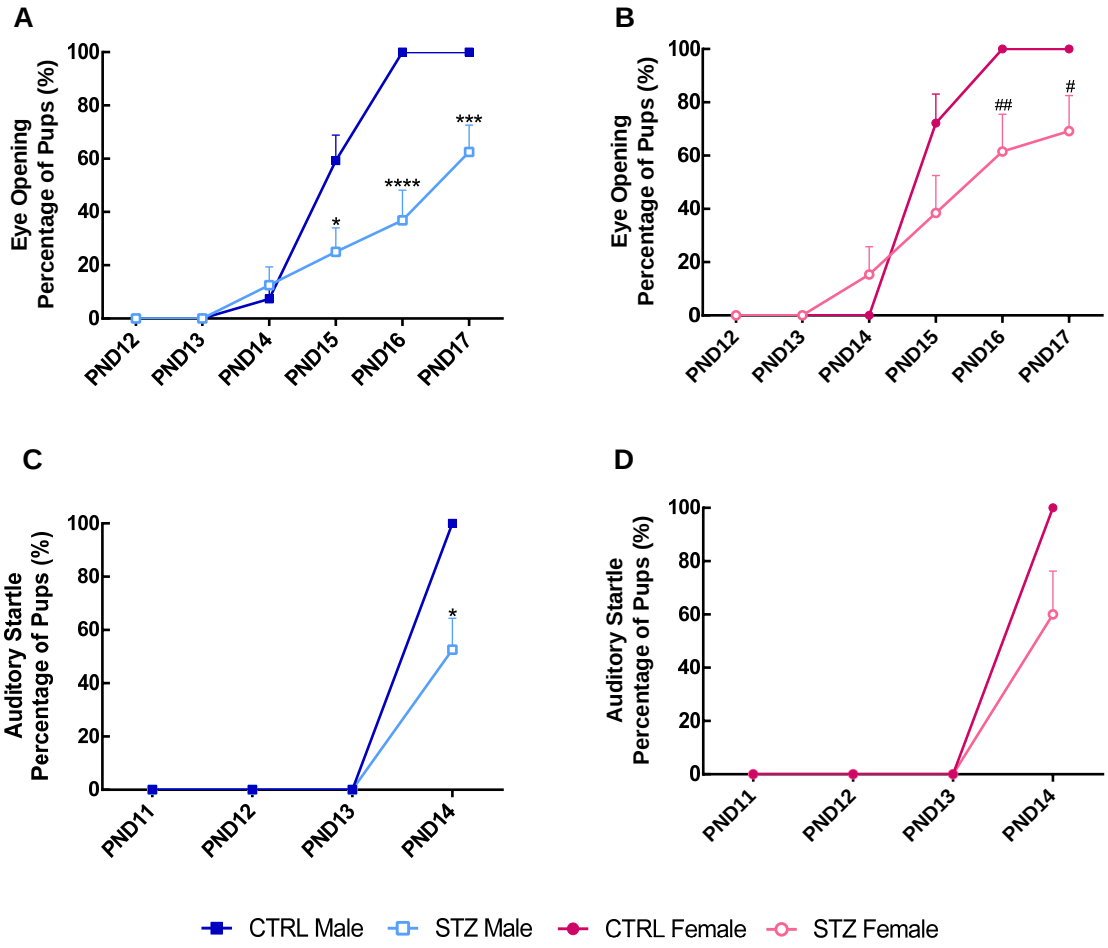


Figure 24. Maternal diabetes induces a delay in male and female offspring's eye opening as well as in the achievement of auditory startle response | Results regarding diabetic dams´ offspring (STZ) and control (CTRL) ones are presented as the mean ± SEM. Left panel shows results for male offspring (blue) and the right panel shows results for female offspring (pink). **(A, B)** Pups were evaluated for their day of eye opening from PND12-PND17. Pups from 5 independent litters from both control and diabetic dams (n=13-27) were used. **(C, D)** Pups were tested for their auditory startle capability from PND11-PND14. Pups from 2 independent litters from control and 4 from diabetic Dams (n=5-19) were used. Statistical analysis was assessed with Student's *t*-test for each time point; *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ compared with male control group; #$p < 0.05$, ##$p < 0.01$, compared with female control group.

tendency for delay in eye opening compared CTRL (PND15 – CTRL♀: 72.2 ± 10.9 %; STZ♀: 38.5 ± 14.0 %; $p = 0.063$), and at PND16 and PND17 percentage of STZ males with eyes opened was statistically significantly decreased comparing with CTRL (PND16 – CTRL♀: 100.0 ± 0.0 %; STZ♀: 61.5 ± 14.0 %; $p < 0.01$ | PND17 – CTRL♀: 100.0 ± 0.0 %; STZ♀: 69.2 ± 13.3 %; $p < 0.05$). All values for percentage of pups with eye open are summarised in Supplementary Data (Table 13).

The percentage of pups (PND11-PND14) with auditory startle response was assessed (Figure 24, C; D). For male offspring at PND11-PND13, no significant differences were observed but at PND14 STZ offspring showed statistically significant lower response than CTRL offspring (PND14 – CTRL♂:100.0 ± 0.0 %; STZ♂: 52.6 ± 11.8 %; $p < 0.05$). For female offspring at PND11-PND13 no statistical differences were observed, nonetheless, at PND14 we could observe a tendency for STZ offspring to have a lower response than CTRL offspring (PND14 – CTRL♀: 100.0 ± 0.0 %; STZ♀: 60.0 ± 16.3 %; $p = 0.082$). All values regarding auditory startle test are summarised in Supplementary Data (Table 14).

4.3. Late Infancy Behaviour Tests

4.3.1. Maternal diabetes does not induce changes in male and female offspring's locomotion

Locomotion was assessed at PND20 in the OPF by placing animals on an open arena and leaving them to freely explore the space for 5 min. No statistically significant differences between STZ and CTRL offspring regarding distance travelled (m) (Figure 25, C; D), and mean speed (m/s) (Figure 25, E; F) were observed in both genders analysed separately. As an indicator of anxious-like behaviour, the time in the centre (s) (Figure 25, G; H), and the number of entries in the centre (Figure 25, I; J), between STZ and CTRL offspring were evaluated. No changes were detected in the parameters analysed. Time in the periphery and number of entries in the periphery also did not demonstrate any significant differences (data not shown).

Interestingly, both CTRL and STZ female pups showed a tendency to spend more time in the centre as well as increased entries in the centre compared with males as can be observed in the illustrative images for each experimental group average occupancy plot (Figure 25, A; B).

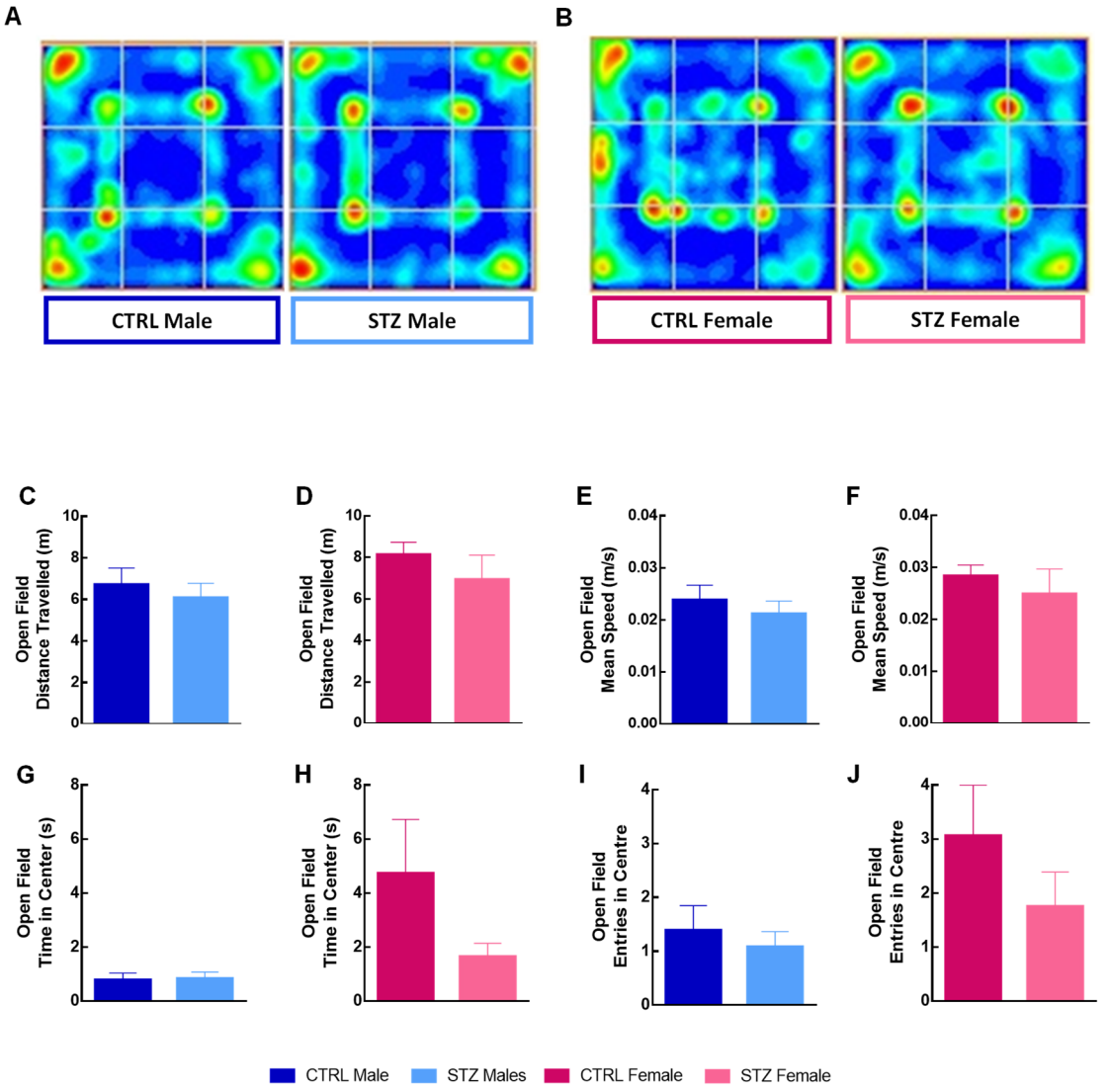


Figure 25. Maternal diabetes does not induce changes in male and female offspring's locomotion | Results regarding diabetic dams´ offspring (STZ) and control (CTRL) ones are presented as the mean ± SEM. Results are shown for male offspring (blue) and for female offspring (pink) side by side of each parameter evaluated. **(A, B)** Animals were tested for exploration in the open field at PND20 and average occupancy plot of the animals' centre for each test group were obtained Pups from 5 independent litters from both control and diabetic dams (n=13-24) were used. **(C, D)** Animals distance travelled in the open field (m) at PND20 was assessed. Pups from 5 independent litters from both control and diabetic dams (n=13-24) were used. **(E, F)** Animals mean speed in the open field (m/s) at PND20 was assessed. Pups from 5 independent litters from both control and diabetic dams (n=13-24) were used. **(G, H)** Animals' time spent in the centre in the open field (s) at PND20 was assessed. Pups from 5 independent litters from both control and diabetic dams (n=12-23) were used. **(I, J)** Animals' number of entries in the centre in the open field (s) at PND20 was assessed. Pups from 5 independent litters from both control and diabetic dams (n=12-24) were used. Statistical analysis was assessed with Student's *t*-test; no statistically significant changes were observed.

4.3.2. Maternal diabetes induces changes in female offspring anxious-like behaviour

Anxious-like behaviour was assessed at PND21 by EPM test. Animals were left for 5 min on an elevated plus shape apparatus to explore the open and/or closed arms. Separate analysis of male and female offspring did not reveal any statistically significant differences between STZ and CTRL offspring for the time spent in the open arms (Figure 26, A; B) The analysis of the number of entries in the open arms, revealed no significant differences for males (Figure 26, C), however for females (Figure 26, D), STZ offspring significantly entered less in the open arms than CTRL ones (PND21 – CTRL♀: 7.5 ± 0.8 s; STZ♀: 5.1 ± 0.6 s; $p < 0.05$). Time and number of entries in the closed arms did not show significant differences (data not shown).

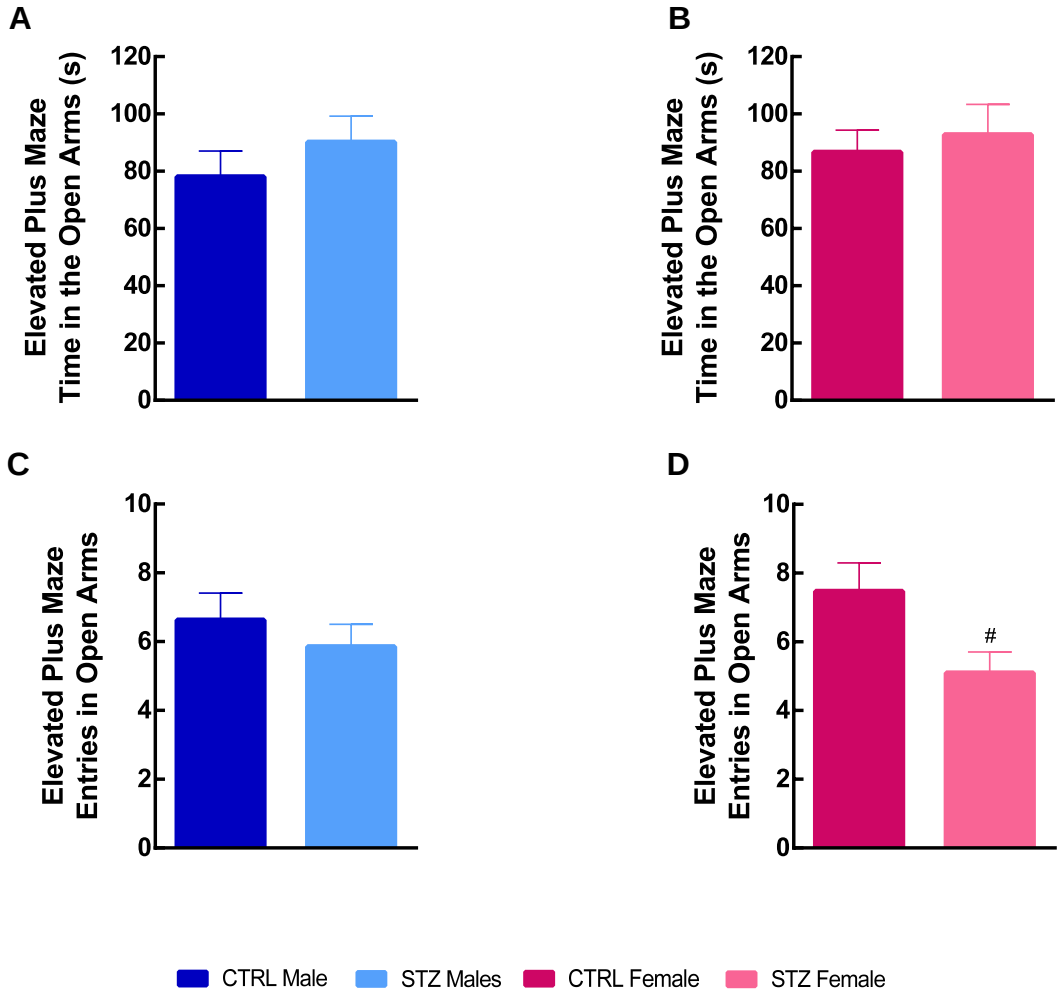


Figure 26. Maternal diabetes induces changes in female offspring anxious-like behaviour | Results regarding diabetic dams´ offspring (STZ) and control (CTRL) ones are presented as the mean ± SEM. Left panel shows results for male offspring (blue) and the right panel shows results for female offspring (pink). **(A-B)** Animals were tested in the elevated plus maze for their time in the open arm at PND21. Pups from 5 independent litters from both control and diabetic dams (n=11-21) were used. **(C, D)** Animals were tested in the elevated plus maze for their number of entries in the open arm at PND21. Pups from 5 independent litters from both control and diabetic dams (n=10-21) were used. Statistical analysis was assessed with Student's *t*-test; #$p < 0.05$, compared with female control group.

Is noteworthy to mention that 2 CTRL♂, 2 STZ♂ and 2 STZ♀ jumped from the EPM platform to the ground and were therefore excluded from the EPM.

4.3.3. Maternal diabetes impairs offspring's novel object recognition

To evaluate recognition memory the NOR test was performed. During the familiarisation trial, both CTRL and STZ should explore both objects, and when exploring should not show preference for a particular one. Animals that did not follow this premises were excluded from the NOR testing. Due to a low number of animals per group, after animal exclusion based on the premises aforementioned, the analysis was performed regardless of gender, joining the data from both male and female for each condition. CTRL and STZ offspring did not explore differently the familiar and novel objects (Figure 26, A) but we could observe a tendency to CTRL animals exploring more the novel object, whereas the STZ offspring to explore less as assed by the recognition index (Figure 26, B). Interestingly, the time of exploration of the novel object by CTRL and STZ offspring was statistically different (PND21 – CTRL♂♀: 22.7 ± 2.3 s; STZ♂♀: 13.8 ± 2.6 s; $p < 0.05$).

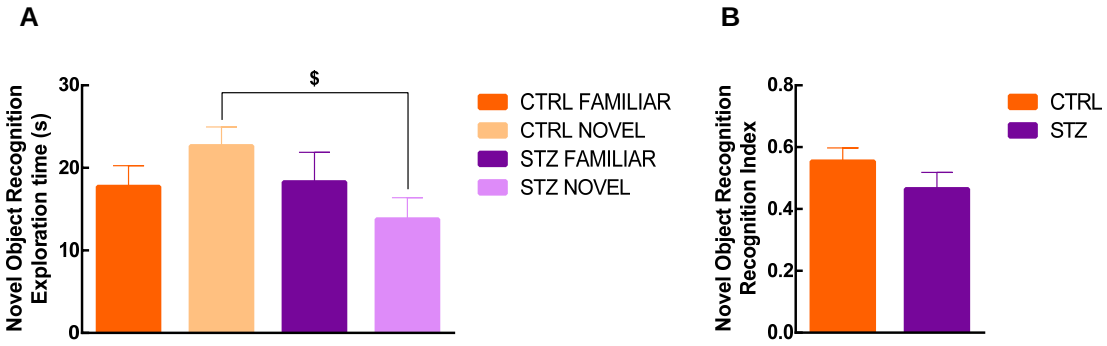


Figure 27. Maternal diabetes impairs offspring's novel object recognition | Results regarding diabetic dams´ offspring (STZ) (purple) and control (CTRL) (orange) ones are presented as the mean ± SEM. Results are shown regardless of gender. **(A)** Animals' were tested in the novel object recognition test for their time of exploration (s) of familiar (light orange/purple) and novel (dark orange/purple) objects at PND21. Pups from 5 independent litters from both control and diabetic dams (n=10-14) were used. **(B)** Animals' were tested in the novel object recognition test for their Novel object recognition index at PND21. Pups from 5 independent litters from both control and diabetic dams (n=10-14) were used. Statistical analysis was assessed with Student's *t*-test; $p < 0.05, comparing exploration of the novel object of control with diabetic offspring.

4.4. Molecular Alterations

4.4.1. Maternal diabetes does not induce changes in male and female offspring's synapsyn-1 and PSD-95 content in the hippocampus

We evaluated the protein levels of synapsin-1 by western blot in total hippocampal extracts (Figure 28, A; B) and hippocampal synaptosomes (Figure 28, C; D). No significant differences in this presynaptic marker were observed for both male and female offspring comparing CTRL with STZ. As a postsynaptic marker, we evaluated the expression levels of PSD-95 by western blot in total hippocampal extracts (Figure 28, E; F) and synaptosomes (Figure 28, G; H), and also no significant differences were observed for both males and females offspring.

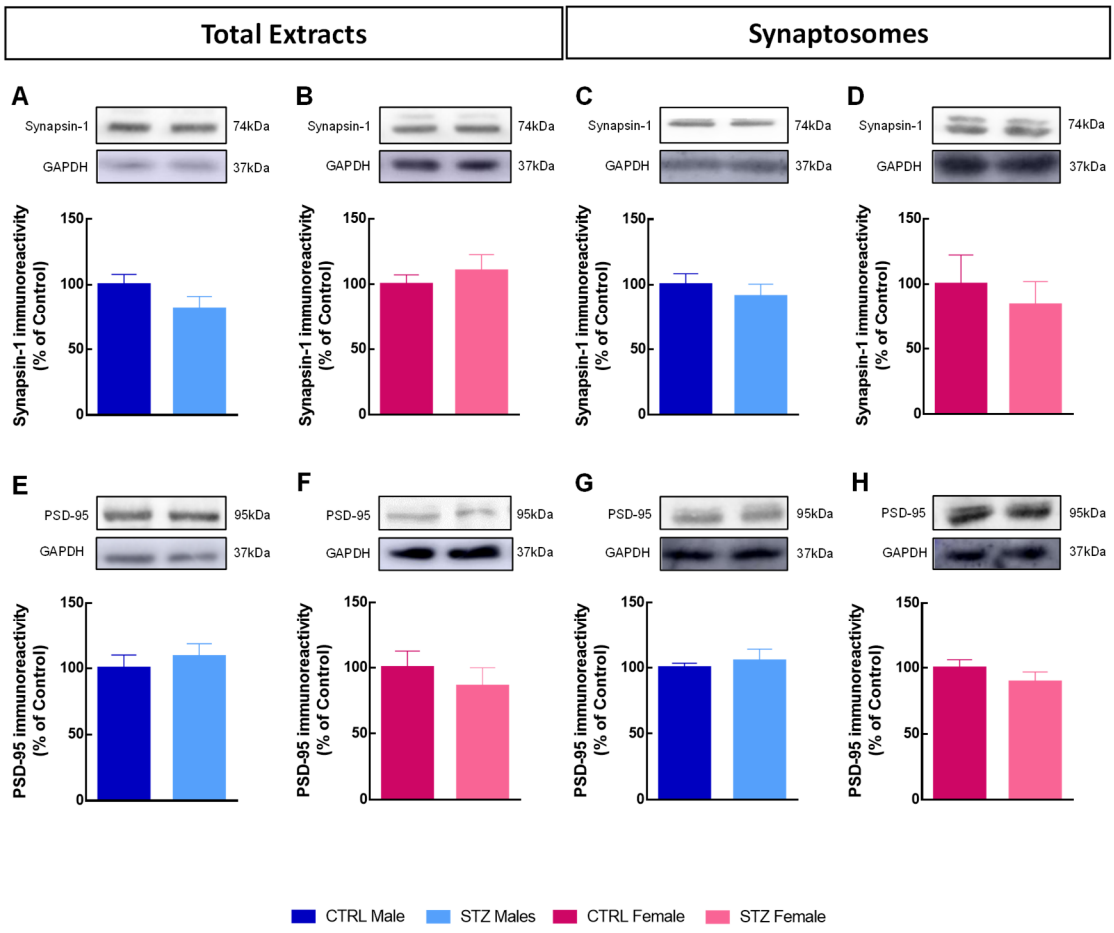


Figure 28. Maternal diabetes does not induce changes in male and female offsprings' synapsyn-1 and PSD-95 content in the hippocampus | Results regarding diabetic dams´ offspring (STZ) and control (CTRL) ones are presented as the mean ± SEM. Left panel shows results regarding total extracts and the right panel shows results regarding synaptosomes derived from hippocampal samples from male offspring (blue) and from female offspring (pink) side by side of each protein evaluated. **(A-D)** Protein levels of synapsin-1 of STZ offspring normalized to control at PND21. Pups from 5 independent litters from both control and diabetic dams (n=6-11) were used. **(E-H)** Levels of PSD-95 normalized to control at PND21. Pups from 5 independent litters from both control and diabetic dams (n=6-9) were used. Statistical analysis was assessed with Student's *t*-test; no statistically significant changes were observed.

4.4.2. Maternal diabetes does not induce changes in male and female offsprings' KIF1A and synaptophysin content in the hippocampus

Protein levels of KIF1A, a motor protein, shown to transport synaptic vesicles containing synaptophysin (Yonekawa et al. 1998; Okada et al. 1995), were evaluated by western blot in total hippocampal extracts (Figure 29, A; B), as well as in hippocampal synaptosomes (Figure 29, C; D), and no significant differences could be noted for males or females comparing both experimental groups. Synaptophysin (synaptic marker) protein levels were evaluated by western blot in hippocampal total extracts (Figure 29. E; F) and synaptosomes (Figure 29, G; H), and no significant differences were observed comparing males or females of both experimental groups.

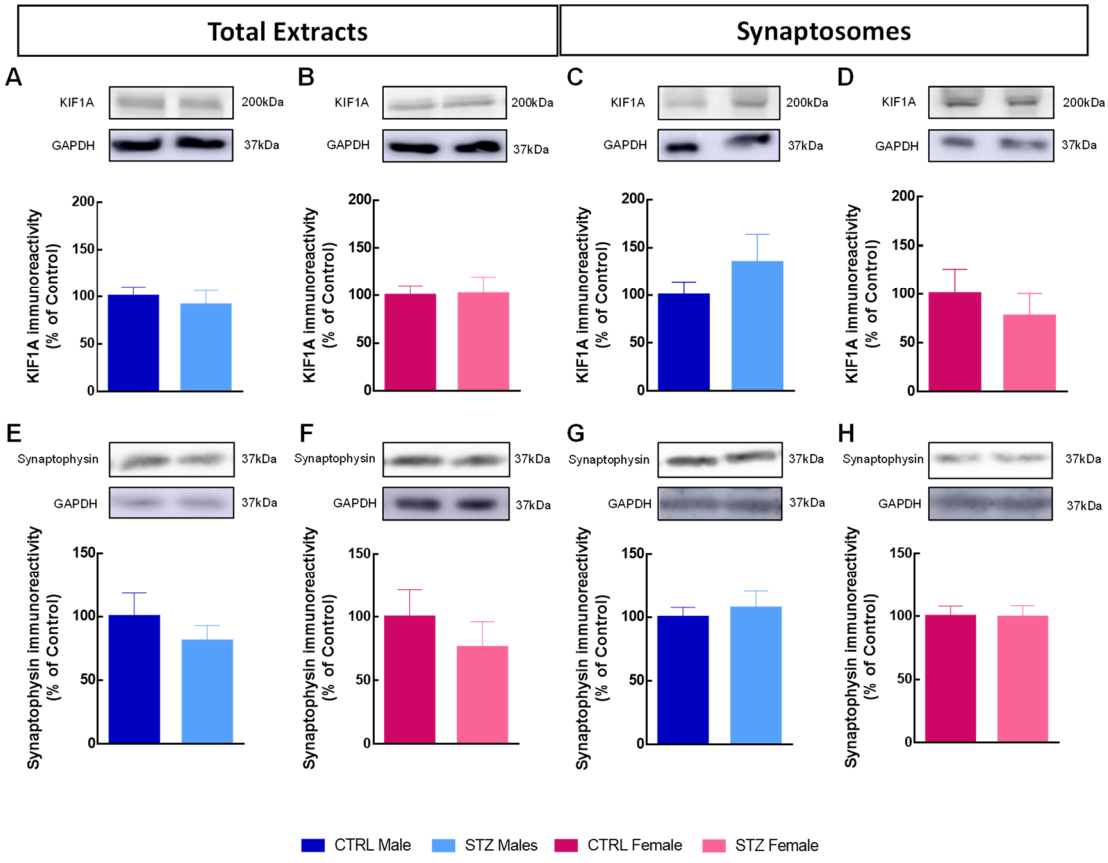


Figure 29. Maternal diabetes does not induce changes in male and female offsprings' KIF1A and synaptophysin content in the hippocampus | Results regarding diabetic dams´ offspring (STZ) and control (CTRL) ones are presented as the mean ± SEM. Left panel shows results regarding total extracts and the right panel shows results regarding synaptosomes derived from hippocampal samples from male offspring (blue) and from female offspring (pink) side by side of each protein evaluated. **(A-D)** Protein levels of KIF1A of STZ offspring normalized to control at PND21. Pups from 5 independent litters from both control and diabetic dams (n=6-10) were used. **(E-H)** Levels of synaptophysin normalized to control at PND21. Pups from 5 independent litters from both control and diabetic dams (n=6-11) were used. Statistical analysis was assessed with Student's *t*-test; no statistically significant changes were observed.

4.4.3. Maternal diabetes does not induce changes in male and female offsprings' VGluT-1 content in the hippocampus

The content of VGluT-1, which is a vesicular glutamate transporter involved on glutamate uptake into synaptic vesicles, was measured by western blot in total extracts (Figure 30, A; B) and synaptosomes (Figure 30, C; D) from CTRL and STZ hippocampi. Regarding VGluT-1 protein levels no differences were observed in males and females comparing CTRL and STZ offspring.

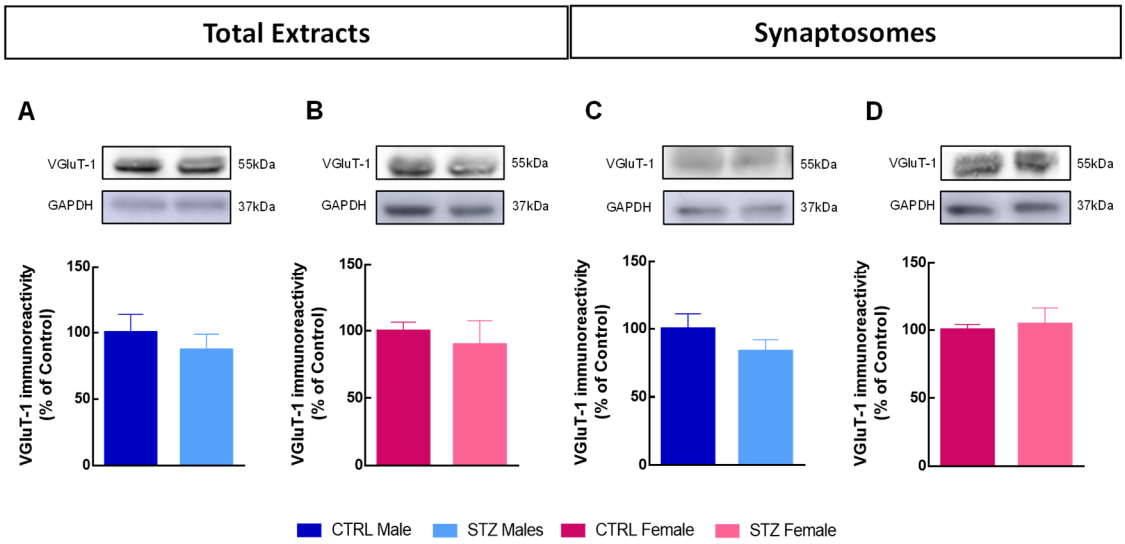


Figure 30. Maternal diabetes does not induce changes in male and female offsprings' VGluT-1 content in the hippocampus | Results regarding diabetic dams´ offspring (STZ) and control (CTRL) ones are presented as the mean ± SEM. Left panel shows results regarding total extracts and the right panel shows results regarding synaptosomes derived from hippocampal samples from male offspring (blue) and from female offspring (pink) side by side of each protein evaluated. **(A-D)** Protein levels of VGluT-1 of STZ offspring normalized to control at PND21. Pups from 5 independent litters from both control and diabetic dams (n=6-10) were used. Statistical analysis was assessed with Student's *t*-test; no statistically significant changes were observed.

4.4.4. Maternal diabetes does not induce changes in male and female offsprings' AMPA receptors' subunit content in the hippocampus

The AMPA receptor subunits (GluA1, GluA2 and GluA4) protein levels were measured by western blot.

GluA1 content in male hippocampal total extracts (Figure 31, A) and synaptosomes (Figure 31, C) showed no differences comparing CTRL and STZ offspring. However, in females, although GluA1 protein levels did not differ significantly in hippocampal synaptosomes (Figure 31, D), in total extracts (Figure 31, B) a clear tendency could be

observed for decreased content in STZ offspring compared to CTRL (PND21 – CTRL♀:100.0 ± 16.8 %; STZ♀: 58.0 ± 12.6 %; p = 0.080).

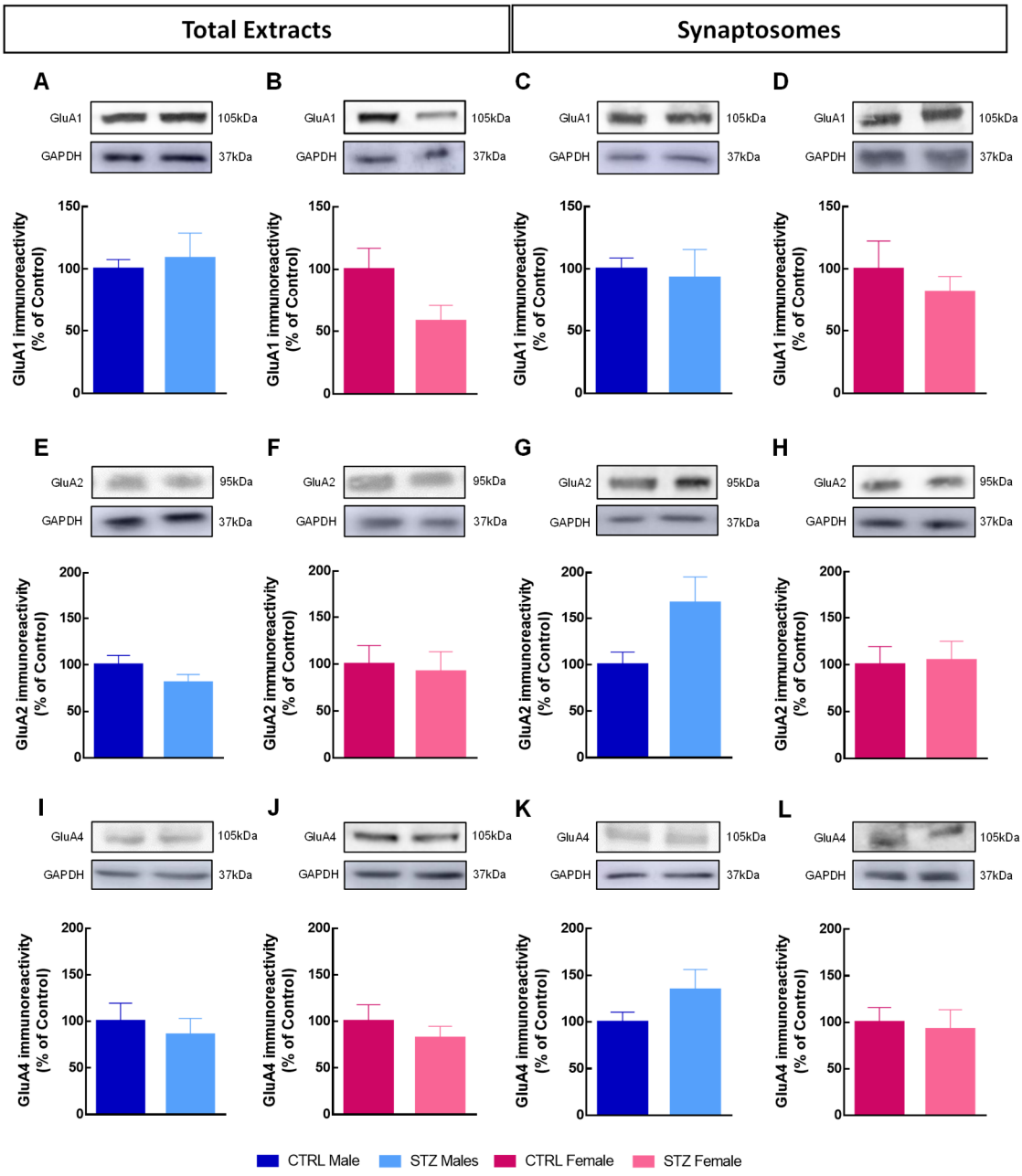


Figure 31. Maternal diabetes does not induce changes in male and female offsprings' AMPA receptors' subunit content in the hippocampus | Results regarding diabetic dams´ offspring (STZ) and control (CTRL) ones are presented as the mean ± SEM. Left panel shows results regarding total extracts and the right panel shows results regarding synaptosomes derived from hippocampal samples from male offspring (blue) and from female offspring (pink) side by side of each protein evaluated. **(A-D)** Protein levels of GluA1 of STZ offspring normalized to control at PND21. Pups from 5 independent litters from both control and diabetic dams (n=6-11) were used. **(E-H)** Levels of GluA2 normalized to control at PND21. Pups from 5 independent litters from both control and diabetic dams (n=6-11) were used. **(I-L)** Levels of GluA4 normalized to control at PND21. Pups from 5 independent litters from both control and diabetic dams (n=4-9) were used. Statistical analysis was assessed with Student's *t*-test; no statistically significant changes were observed.

The protein levels of GluA2 in female hippocampal total extracts (Figure 31, F) and synaptosomes (Figure 31, H) remained unchanged when comparing both experimental groups. However, in males, although GluA2 protein levels did not differ significantly in hippocampal total extracts (Figure 31, E), in synaptosomes (Figure 31, G) a clear tendency to increased levels could be observed (PND21 – CTRL♀: 100.0 ± 13.6 %; STZ♀: 166.4 ± 29.0 %; p = 0.070).

Finally, the protein levels of GluA4 were evaluated in both male and female in hippocampal total extracts (Figure 31, I; J) and synaptosomal extracts (Figure 31, K; L) by western blotting and no changes were detected when comparing CTRL and STZ offspring.

4.5. Hippocampal Structural Alterations

4.5.1. Maternal diabetes does not induce changes in the thickness of the CA1, CA3 and DG nuclear layers of the male and female offspring's hippocampus

We evaluated the thickness of the nuclear layers of CA1, CA3 and DG subregions of the dorsal hippocampus by cresyl violet staining (Figure 32, A). No changes in layer

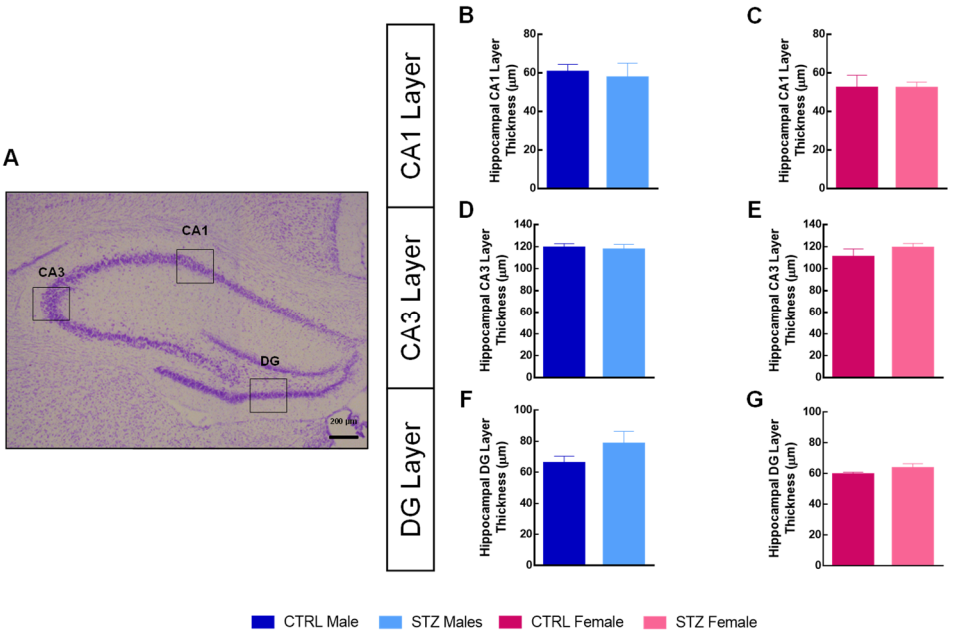


Figure 32. Maternal diabetes does not induce changes in the thickness of the CA1, CA3 and DG nuclear layers of the male and female offspring's hippocampus | Results regarding diabetic dams´ offspring (STZ) and control (CTRL) ones are presented as the mean ± SEM. Left panel shows results for male offspring (blue) and the right panel shows results for female offspring (pink). **(A)** Hippocampal slice stained with cresyl violet representing subregions of interest. **(B, C)** Hippocampal CA1 layer thickness (µm) of animals at PND21. Pups from 5 independent litters from both control and diabetic dams (n=4-5) were used. **(D, E)** Hippocampal CA3 layer thickness (µm) of animals at PND21. Pups from 5 independent litters from both control and diabetic dams (n=4-5) were used. **(F, G)** Hippocampal DG layer thickness (µm) of animals at PND21. Pups from 5 independent litters from both control and diabetic dams (n=4-5) were used. Statistical analysis was assessed with Student's *t*-test; no statistically significant changes were observed. Scale bar: 200µm.

thickness were observed in male and female offspring of diabetic dams compared to CTRL male and female offspring, respectively, in the CA1 (Figure 32, B; C), CA3 (Figure 32, D; E) and DG (Figure 32, F; G).

Chapter 5

5. Discussion

5. Discussion

Diabetes during pregnancy has been associated with increased risk of neurodevelopmental disorders in the offspring. Experimental studies indicates that it induces modifications in offspring hippocampal structure and function (DeRegnier et al. 2000; Nelson et al. 2000; Deboer et al. 2005; Vafaei-Nezhad et al. 2016; Golalipour et al. 2012; Chandna et al. 2015; Ramanathan et al. 2000; Vuong et al. 2017). Nevertheless, the exact mechanisms by which *in utero* diabetic environment affects the offspring hippocampus remain to be defined.

In this work a longitudinal study in the context of maternal diabetes was performed to assess their consequences on offspring development and memory processes, combining cellular and molecular techniques with neurobehavioural tests. Since diabetic intrauterine environment may also exert differential effects on offspring neurodevelopment based on gender (Kinney et al. 2003; Chandna et al. 2015), both sexes were analysed independently, expecting to find a gender-specific susceptibility.

In this work we monitored animals' weight gain and observed that both male and female pups of diabetic dams show decreased weight comparing, respectively, with male and female pups of control dams, showing, therefore, a delayed development or microssomia. Despite the animal model used in this work (injection of 45mg/Kg STZ, ip) is described to induce moderate diabetes (Vafaei-Nezhad et al. 2016), our results are in accordance with what is described for severe cases of diabetes (Van Assche et al. 2001). According to literature, the magnitude of maternal diabetes (mild or severe) has a differential impact on the foetal development in the rodent models (Van Assche et al. 2001; Jawerbaum & White 2017; Kiss et al. 2012). Mild maternal diabetes has been described to leave the foetus exposed to increased amounts of glucose that permeate the placenta. This hostile environment makes the foetus adapt through increasing insulin production and action. The foetus extra insulin requirements enhance development of the Langerhans islets resulting in hypertrophy of the endocrine pancreas and hyperplasia of the β-cell which may lead to increased weight or macrosomia (Van Assche et al. 2001). Therefore, after birth, it is described an neonatal hypoglycaemia attributed to maternal hyperglycaemia, which in turn induces foetal hyperinsulinemia (Weintrob et al. 1996; Persaud 2007). However, in severe maternal diabetes, the increased glycaemia observed is too great and leads to an intense activity from the foetuses β-cell that deplete their stores as they are not able to biosynthesize insulin at the rate that it is secreted (Kervran et al. 1978; Van Assche et al. 2001), leading to hypoinsulinemia and to a reduction in foetal glucose uptake. As a consequence growth of foetal protein mass and foetal protein synthesis is consistently lower

than in controls (Canavan & Goldspink 1988). Postnatal development of the microsomic pups born to severely diabetic mothers is described to be retarded, and the animals remain small up to adulthood (Leona Aerts et al. 1990).

Keeping these features in mind our results seem not to accompany the mild diabetes description, but seem to follow the more severe maternal diabetes features. Glycaemia values were tendentiously higher in STZ offspring compared to CTRL, at birth and PND7, for both males and females exposed to *in utero* maternal diabetes, reaching statistical significance in some cases. At PND21 the higher glycaemia reported in STZ offspring in the first postnatal week is no longer evident. Even though we found significant differences in offspring's glycaemia after birth we do not report hyperglycaemic offspring at PND21, despite the fact that several studies report hyperglycaemic offspring from birth to PND21 (Chandna et al. 2015; Hami, Vafaei-Nezhad, Ghaemi, et al. 2016; Hami, Vafaei-Nezhad, Haghir, et al. 2016; Vafaei-Nezhad et al. 2016; Ramanathan et al. 2000).

Regarding plasma insulin, right after birth (PND0), STZ female offspring showed a tendency for lower insulin levels than CTRL. For later timepoints after birth (PND7 and PND21) no significant alterations could be noticed. This is also in agreement with a case of severe maternal diabetes because, as previously referred, β-cells become depleted due to the *in utero* foetal exposure to the strong hyperglycaemic environment (Kervran et al. 1978; Van Assche et al. 2001) and only after birth β-cells are able to restore their insulin pool and respond with effectiveness to circulating levels of glucose. It is important to notice that the number of animals per group should be increased, namely at PND0 in order to evaluate if the different levels of insulin obtained reach statistical significance.

Maternal diabetes did not induce gender-specific susceptibility in terms of weight, glycaemia and plasma insulin levels.

Dams' weight and glycaemia were monitored throughout the study to confirm that CTRL dams were normoglycaemic and STZ-injected dams were diabetic. Interestingly, at GD21, results revealed that pregnant STZ dams and CTRL ones did not differ in terms of weight while the literature describes, specifically for severely diabetic rat, that during pregnancy fat deposits are mobilized, and animals hardly gain weight during pregnancy (L Aerts et al. 1990). Therefore, in this parameter analysed, our animal model is not in accordance with what is described for more severe cases of maternal diabetes. Of note, we did not find any statistically significant differences in the number of pups born from diabetic dams comparing with control ones (data not shown), which could influence dams' weight at GD21.

Diabetes during pregnancy has been implicated in developmental susceptibility to psychomotor delays in early ages in humans (Rizzo et al. 1995). Therefore, to evaluate if maternal diabetes induces developmental alterations in the offspring and possible delayed

development, we applied a battery of tests to evaluate acquirement of reflexes, strength and pup locomotion, and to assess the achievement of physical developmental milestones.

In some experimental studies, after birth the litters from STZ-injected mothers are cross-fostered to control dams (Hami, Vafaei-Nezhad, Ghaemi, et al. 2016; Kinney et al. 2003), in order to reduce the maternal diabetes impact only to gestation and not taking into account the lactation period. However, in this work we decided not to cross-foster the offspring born from STZ-injected mothers to control dams because early-life adoption could cause significant stress to the pups (Darnaudéry et al. 2004), and also because a previous work has shown that intrauterine glycaemic state is the fundamental factor for neurodevelopment and not the quality of milk during lactation period (Kinney et al. 2003).

In order to evaluate trunk control and vestibular system deficits, we used righting reflex tests (Feather-Schussler & Ferguson 2016) and both male a female STZ offspring demonstrate a slight tendency to have deficits. Negative geotaxis reaction test evaluating motor coordination, balance and also vestibular system deficits demonstrated a stronger effect of maternal diabetes on offspring performance. In terms of cliff aversion, which relies on rats inherent fear of falling and ability to go towards safety, testing for motor control (Feather-Schussler & Ferguson 2016), STZ animals present impairments as well. An overall delay in development of reflexes was observed in the STZ offspring, even though for each test the differences are not reiterated throughout all the testing days. These tests are used as an indication of maturation of somatosensory, vestibular and/or proprioception function and our findings are suggestive of a delayed maturation (neurogenesis and/or myelination) of these systems or hindered functional organization of the complex synaptic circuitry that is underlying these reflexes in the brain. Furthermore, it seems that an effect of gender might be present because males appear to be more affected by maternal diabetes than females, though it could also be a reflex of the lower number of females pups studied compared to males. In wire suspension test for forelimb strength (Feather-Schussler & Ferguson 2016), STZ offspring also seem to have less strength in forelimbs. Locomotion tests for the acquisition of mobility reveal that STZ offspring show a delay in development as well. In nest seeking test, we measured the time pups took to reach their home bedding and, we found that pups from STZ-diabetic dams have impaired performance comparing with CTRL ones. For males there was some consistency for STZ having worse performance, even though not throughout all time points and for females there was one day when, in fact, STZ offspring performed significantly better than CTRL ones. As already described, nest seeking test is dependent on many different factors, namely locomotion which was impaired in the case of STZ offspring. Since performance in nest seeking test is also dependent on locomotion to achieve the home bedding, components evaluated in this test, like olfaction, mother-pup relation and discrimination of home bedding may be biased

by impaired locomotion. Also, an effect of bedding age may also influence pup performance in this test, since STZ animals have more frequent urination and bedding is exchanged more frequency than CTRL ones and therefore may be a factor to take into account. For better understanding of maternal-pup relationship, it would be important, for example, to look for differences in pup ultrasonic vocalizations after mother separation, a tool to monitor early communicative behaviour, which when impaired is a reliable indicator of an aversive affective state of pups (Baharnoori et al. 2012). In fact, a study has described that the offspring of STZ-injected rats PND4 and PND6, emitted higher numbers of ultrasound calls compared to control offspring (Johansson et al. 1991).

Remarkably all throughout this developmental evaluation of the influence of maternal diabetes in the offspring seems to show more marked deficits beginning at PND8. So PND8 seems to apparently be a day of particular importance during development. In fact PND8 is the time auditory system of infant rat begins to functionally develop, is the day at which the vestibular system becomes largely adult-like, is also the day at which animals show the spatial awareness to orientate themselves towards the home nest, and additionally is the day pups gain motor ability to pivot (Tan et al. 2017; Wills et al. 2014).

Moreover, the evaluation of the eye opening day demonstrated that STZ offspring had a clear delay in the achievement of this developmental milestone, which is in disagreement with results obtained in a previous study using an animal model of neonatal mild diabetes (Kiss et al. 2012). Kiss et al. used Wistar rats injected at birth with 100 mg/kg STZ subcutaneously (that produces a mild hyperglycaemia) and then at PND90 mated them and analysed their offspring. Although this model is widely different from ours, authors reported an advance in eye opening correlating this finding with insulin and other growth factors levels that are a prominent determinant of foetal development. They propose that there could be increased growth factor levels and in our case could be the opposite as decreased insulin and growth factors levels can at least in part explain a delay in the achievement of this physical milestone. Further analyses to measure rat pup insulin levels could shed some light on the reason behind this result.

We were not able to evaluate ear opening day and therefore used instead the auditory startle test to evaluate auditory system development. We observed, at least, in males a delay in such reflex that was not significant in females. This delay could once more denote a possible indication of delayed maturation of the auditory system and vestibular function.

Overall our results indicate that maternal STZ-induced diabetes leads to abnormal development of the offspring in early postnatal days. It is worth mentioning that, as far as we are concerned, this was the first time that a longitudinal study on the achievement of developmental milestones was performed in the context of maternal diabetes and their consequences to the offspring.

Several evidences show that maternal diabetes can induce cognitive impairment in both humans (DeRegnier et al. 2000; Nelson et al. 2000) and animal models (Ramanathan et al. 2000; Kinney et al. 2003; Vuong et al. 2017). Additionally, epidemiologic studies have shown that the offspring of women who experienced diabetes mellitus during their pregnancies are seven times more likely to develop schizophrenia, compared with those who were not exposed to diabetes in pregnancy (Van Lieshout & Voruganti 2008). Maternal pre-existing type 2 diabetes, as well as exposure to gestational diabetes diagnosed at 26 weeks' gestation was associated with risk of autism spectrum disorders in offspring (Xiang 2017). The gestational environment can, therefore, impact foetal brain structure and function and increase long-term susceptibility to neurodevelopmental and neuropsychiatric disorders.

Although not the main aim of this study, we evaluated the performance of CTRL and STZ offspring in OPF and EPM tests since anxious-like behaviour can also impact rat cognition. OPF test was firstly used to evaluate locomotion and secondly to evaluate ambulation in the central area. In fact, animals that have an anxious-like behaviour when exposed to a new environment present low ambulation, especially in the central zone (Sestakova et al. 2013). Our findings, at PND20, do not point towards changes in the distance travelled and mean speed between STZ and CTRL offspring, showing that there are no impairments concerning locomotion, even though through early postnatal days we observed that STZ offspring had developmental motor and locomotor deficits. Remarkably our results regarding the OPF seem to not follow what has been described in a previous work (Ramanathan et al. 2000). In a pregestational STZ-induced diabetic rat model, authors report increased ambulance (number of OPF squares crossed) of the offspring of diabetic dams, although not making gender distinction. Our analysis was in terms of distance travelled and mean speed while theirs was in number of squares crossed, nevertheless, results should be relatable. Possible reasons for such difference could be related to the fact that our analysis was performed in animal at PND20 and theirs was performed in the offspring with 8 weeks whose mothers were injected with 50 mg/kg rather than 45 mg/kg as we used, furthermore their rat strain was Foster albino and not Wistar like we used.

To further explore if STZ animals presented an anxious-like behaviour we performed the EPM test and no differences were found regarding the time spent in the open arms for both male and female offspring from both experimental groups on EPM at PND21. Nevertheless, STZ females enter the open arms significantly fewer times than CTRL ones, pointing toward a possible anxious-like behaviour effect of maternal diabetes. EPM results could be correlated with the tendency observed for STZ females in the OPF for decreased entries and time spent in the central area of the arena also suggestive of an anxious-like behaviour. Regarding our findings related to anxious-like behaviour, others have reported

such results although not taking into account gender differences. Ramanathan et al. (2000) reported that hyperglycaemia in pregnancy caused offspring (8 week of age) to display anxious-like behaviour both in EPM and OPF (Ramanathan et al. 2000), which even though the authors did not distinguish gender in their analysis, is in agreement with our results, because the anxious-like behaviour reported may be attributed to female offspring since gender was not taken into account, as we report. Interestingly, in a gestational diabetes animal model, offspring of STZ-injected and STZ-injected treated with insulin dams dwelled more in the open arms of the EPM showing a disinhibition compared to control ones (Chandna et al. 2015). The contrasting results reported may reside on several differences in the model used since the authors induced diabetes during pregnancy at GD13 by 50mg/kg STZ ip injection, while we administered it before pregnancy. Moreover, STZ itself could cross the placenta and therefore interfere with foetal development and behaviour. Nevertheless, the authors claim that this may not be occurring, since STZ is cleared from dams circulation after 6h and since pup weight was unaltered at PND0 and showed no evidence of hyperglycaemia at PND7 and PND40 further suggesting that STZ does not directly impact foetal development (Chandna et al. 2015). Moreover, a study performed at PND60 on both male and female offspring of Sprague-Dawley rats injected with 30-35 mg/kg STZ, ip, did not present alterations in anxious-like behaviour assessed by EPM test relative to control ones (Kinney et al. 2003). Once more differences can relate to the underlying differences in the model of maternal diabetes implemented and postnatal day assessed.

Studies have suggested differences in behaviour related to age that manifest themselves particularly as an increasing drive to explore new places and reduced capacity of risk assessment (Doremus et al. 2006). This may explain the fact that some animals jumped from the open arms of the EPM at PND21. In other studies this type of behaviour is described as an indicator of disinhibition or impulsivity and is reported in animals with altered intrauterine environment (VanRyzin et al. 2016). Nevertheless, in our case it is probably not related to maternal diabetes since both CTRL and STZ offspring displayed this behaviour. Moreover comparing adolescent (PND33-PND35) and adult (PND70-PND75) Sprague-Dawley rats, differences were not found in EPM analysis (Doremus et al. 2004), nevertheless caution should be taken when analysing youths as anxiety-related brain structures are undergoing maturation and variations could emerge when comparing results in adolescents and adults animals (Barrera-Bugueño et al. 2017).

Aiming to assess the impact of maternal diabetes on offspring memory we performed the NOR test. The NOR test is based on the innate tendency of rodents to differentially explore novel objects over familiar ones, and a significantly higher exploratory preference reflects good recognition memory, being an indicator of short-term recognition memory. If

animals do not explore the novel object more than the familiar object it is an indication that they display impaired recognition memory. As described in the results section, some animals, both CTRL and STZ, showed a certain degree of disinterest for exploring the objects. Indeed some animals did not explore the objects or explored one object much more time than the other one in the familiarization trial. Therefore, the exclusion of these animals left us with a lower number of animals to maintain gender differentiation in the analysis, and in this case, male and female offspring were analysed together. The analysis of exploration time of novel and familiar object as well as the recognition index, a measure of novel object recognition (Antunes & Biala 2012) in the testing trial, revealed that neither CTRL nor STZ demonstrated to have a statistically significant preference for novel object in relation to the familiar object. However, even though these conventional measures do not show any significant differences we noticed CTRL offspring demonstrate a preference for novel object (as expected) comparing with the STZ offspring which may be an indicator of impaired memory, a finding that needs further study in order to determine if in fact there is impaired cognition. A previous study reported impaired memory evaluated in the NOR, in an animal model of gestational diabetes (Vuong et al. 2017) which goes in accordance with our results that there might be impaired cognitive function in the STZ offspring, even though further confirmation of the preliminary data is required. Chandna et al. (2015) on the other hand, reported that in a gestational diabetes animal model, male offspring did not display deficits in the NOR, and in fact presented increased preference for the novel object in the NOR displacement test (Chandna et al. 2015). The NOR displacement test was performed a week after the conventional NOR and consists of changing the location of one of the objects rather than the object itself and testing, after a 24 h, animals ability to recall the place where the object was. The differences between our results and that of Chandna et al. (2015) may be related to the model used since these authors injected STZ during gestation. Furthermore, as previously mentioned, Chandna et al (2015) only analysed the male offspring, whereas in our work we evaluated male and female offspring. Reinforcing the need for gender differential analysis, Kinney et al. (2003) found that maternal diabetes induces learning deficits only in female long-term memory, but not in working memory tasks (Kinney et al. 2003). In their analysis, Kinney et al. (2003) induced diabetes by injection of 30-35 mg/kg STZ, ip, in Sprague-Dawley females prior to mating and analysed the offspring, both male and female separately at PND60, using the Lasheley III maze and the inhibitory avoidance task to test long-term memory and learning, and the 12-arm radial maze for short-term memory. Once again we emphasise that we are comparing results from adult rats (PND60) with late infant rats (PND21) and for instance, only at PND20 emerge capability of spatial learning, and it has been described that active exploration merely develops around PND21. Furthermore open field as well as object exploration continues to mature between

PND30 and PND90 (Wills et al. 2014; Tan et al. 2017) which may also account for our observation of animals lack of interest in exploring the objects and cautions us to look for more subtle differences between CTRL and STZ offspring, as controls may still have not fully developed their exploration and learning skills.

With this work we intended to correlate the impact of maternal diabetes on offspring memory with molecular changes in the hippocampus as synaptogenesis and neurotransmission. Maternal diabetes did not induce changes in protein levels of synapsin-1 and PSD-95, in both hippocampal synaptosomes and total extracts of STZ offspring. Furthermore, KIF-1A and synaptophysin protein levels also remained unchanged. Altogether these results do not indicate any alterations in terms of synaptic protein content that could lead us to believe that there is an impairment of synaptogenesis in the offspring due to maternal diabetes. In disagreement with our results, others found that diabetes in pregnancy altered the distribution of synaptophysin in several hippocampal subregions and describe a downregulation of this protein's mRNA (Vafaei-Nezhad et al. 2016; Vuong et al. 2017). Nevertheless, these alterations were observed at PND0, 7 and 14, whereas we analysed the hippocampus at PND21, and therefore we cannot exclude that for earlier timepoints a decrease in synaptic proteins content may occur.

Similarly to the other synaptic proteins evaluated in this study, no changes were detected in the levels of VGluT-1, suggesting no alterations in glutamatergic synapses. During gestation, the offspring of a diabetic mother is exposed to high glucose levels that cross the placenta In a previous *in vitro* study from our group, changes in the content and distribution of synaptic and motor protein levels, are found in hippocampal cultures exposed to high glucose (Baptista et al. 2013; Gaspar, Castilho, et al. 2010). Also, in the hippocampus of an STZ-induced diabetic animal model, changes in several synaptic and motor protein were observed (Gaspar, Baptista, et al. 2010; Baptista et al. 2013), as well as changes in basal glutamate release (Baptista et al. 2011). Together these results highlight the negative impact that hyperglycemia may have, affecting neurons and inducing synaptic changes.

AMPA receptors shape synaptic transmission, influence integration and synaptic inputs (Greger et al. 2017). AMPA receptor subunit composition differs in synaptic and extrasynaptic sites and their differential expression shapes excitatory potential synaptic currents (EPSC) (Jacobi & von Engelhardt 2017). Therefore, we looked for alterations in the levels of GluA1, GluA2 and GluA4, AMPA receptor subunits. We did not evaluate GluA3 levels because besides being minimally expressed in relation to GluA1 and GluA2 levels (~10%) (Henley & Wilkinson 2016) there was lack of suitable anti-GluA3 antibody to allow for this subunit level quantification. Overall, we found no statistically significant alterations in AMPA receptor subunits in both female and male STZ offspring hippocampus. Still, some

tendencies were observed, as GluA2 content was slightly increased in male STZ synaptosomes. The majority of AMPA receptors contain the edited form of GluA2 (the predominant expressed form in adult brain) which when incorporated in the receptor render it impermeable to Ca^{2+}-impermeable. GluA2 subunit has a strong effect on AMPA receptor assembly and trafficking and a dysregulation of GluA2 incorporation in the AMPA receptor complex have implications in neuronal damage and disease (Henley & Wilkinson 2016). Additionally, GluA1 protein levels were non-significantly decreased in total extracts, only in female STZ offspring but the alteration was not verified at the synaptic level. GluA1 is dominant in activity-dependent recruitment of the AMPA receptors to the synapse (Henley & Wilkinson 2016) therefore it is possible that the overall decreased levels of GluA1 could lead to impaired recruitment of AMPA receptor to the synapse and consequently animals could have, as a result, impaired response to LTP which would relate to cognitive dysfunction. Furthermore, the apparent changes in these receptor subunits that we report seem to denote a possible gender difference.

Previous results from our group, on a study in the context of diabetic encephalopathy and not diabetes in pregnancy, it was reported unaltered GluA1, GluA2 and GluA4 protein levels in total and cell surface expression of hippocampal neurons exposed to high glucose. In STZ-induced diabetic animals, it was found an increase in GluA1 protein levels at 2 weeks diabetes and a decrease in GluA2 at 4 week diabetes in total hippocampal extracts. In plasma membrane it was found a decrease in GluA2 protein levels at 2 weeks diabetes (Castilho et al. 2012). Altogether, AMPA receptor changes may alter synaptic plasticity, and therefore memory, since AMPA receptors are the prime elements that undergo changes in these events (Greger et al. 2017). These results highlight the importance of electrophysiological evaluation of the impact of maternal diabetes in the hippocampus to unravel the mechanism behind memory impairments in the offspring of diabetic dams.

Regarding our analysis of the integrity of the nuclear layers of the CA1, CA3 and DG subregions, we did not report differences between the nuclear thickness of STZ hippocampal subregions comparing with the CTRL. In a recent study,, the authors performed NeuN immunohistochemistry and measured CA1 neuronal layer reporting similar results, namely that maternal diabetes (diet-induced) doesn't alter total CA1 neuronal layer width, as well as the dense CA1 layer thickness (Vuong et al. 2017). Interestingly, if the offspring besides being exposed *in utero* to maternal diabetes, are also fed with a high fat and sucrose diet, they actually present deranged CA1 neuronal layer showing increased total CA1 width and decreased dense CA1 width (Vuong et al. 2017). Moreover, in the same study, on both offspring from diabetic dams as well as those offspring subjected to high fat and high sucrose diet, synaptophysin levels quantified in CA1 region decreased, being indicative of possible decreased synaptic terminal integrity.

Others have also performed cresyl violet staining to evaluate CA1 and CA3 nuclear layer thickness finding concordant results with ours by observing no differences in the pyramidal cell layer thickness on the offspring at PND21 (Golalipour et al. 2012). Their evaluation was performed also on PND7 offspring and, no differences were observed at this age either. Surprisingly, when counting the number of cells in those layers, they report a reduction of pyramidal cells at both PND7 and PND21 on both subregions analysed. We did not evaluate in our study the number of cells of the hippocampal CA1, CA3 and DG subregions but our results regarding unaltered synaptic protein levels, further suggest that the cell number could be unchanged as well, but this issue needs further analysis to clarify it.

Chapter 6

6. Conclusion

6. Conclusions

Maternal diabetes has been implicated in long-term neurological consequences in the offspring. In fact, the offspring of diabetic mothers have been described to have memory deficits that have been correlated with changes in the hippocampus.

Therefore, in our work, we intended to evaluate the impact of maternal diabetes in memory processes in a late infancy stage and uncover the possible cellular and molecular hippocampal changes that could be the basis of such impairment.

The results presented in this thesis allowed the drawing of the following main conclusions:

- The offspring of diabetic dams were microssomic during the first weeks of life, showing impaired weight gain in relation to control until PND21. This was observed in both male and female offspring. Furthermore in the first week of life, both male and female had higher glycaemia levels than the controls, even though the levels did not reach a state that could be considered hyperglycaemic. These findings, and according to what is described in the literature, lead us to conclude that, in our hands, the streptozotocin dose used in this study to induce diabetes caused a severe diabetic model rather than a moderate one as previously described.

- Early life monitoring of reflex, strength and locomotor development, as well as physical milestones achievement, showed a clear delay in diabetic mother's offspring which appeared to be more pronounced in the male offspring.

- In late infancy, maternal diabetes caused an increase in anxious-like behaviour that was only observed in female offspring.

- Short-term memory of the offspring of diabetic mothers appears to be affected but we were not able to evaluate female and male offspring separately, and therefore we were not able to correlate memory deficits with gender.

- Synaptogenesis does not show signs of alteration since the levels of the synaptic proteins analysed for animals 21 days old remained unchanged.

- Glutamate neurotransmitter uptake into synaptic vesicles does not seem to be impaired in the progeny of diabetic mothers, nevertheless, further studies will be needed to assess possible impairments in glutamate release.

- Protein levels of AMPA receptors subunits GluA1 appears to be decreased in STZ female hippocampus which could be implicated on impairment of recruitment of AMPA receptor to the synapse. GluA2 appeared to be increased in STZ male hippocampal synapses which could be suggestive of alteration in AMPA assembly and trafficking. -

Offspring hippocampal formation structural analysis did not show differences in nuclear layer thickness, therefore not showing indication of neuronal loss due to maternal diabetes.

To conclude, our work provided further insight into the impact that maternal diabetes has in the developing offspring. Further investment in exploring the impact of maternal diabetes in cognitive function using forefront techniques, will give a better insight into the exact mechanisms responsible for the effects of maternal diabetes on cognitive development of the offspring, providing new ways to intervene and thus prevent or modify the neurodevelopmental effects of maternal diabetes. Also, since we were already able to find some indications of a differential impact of maternal diabetes in male and female offspring, further understanding of gender differences in etiology and clinical presentation will be fundamental for the development of pharmacological treatments.

Chapter 7

7. Future Perspectives

7. Future Perspectives

The present work highlights the importance of fully understanding the deleterious effects of maternal diabetes during brain development, also demonstrating the importance of studying gender differences in response to insults during neurodevelopment. To further clarify the mechanisms responsible for the effects of maternal diabetes on cognitive development of the offspring a large integrated study in the context of maternal diabetes and their consequences on offspring memory processes, using *in vivo* forefront techniques, together with cell and molecular biology approaches should be performed in the future.

Several questions remain:

- Does maternal diabetes affect offspring hippocampal neurogenesis and synaptogenesis?

Future studies should address whether diabetes during pregnancy induces changes in offspring neuron formation. For that, neurogenesis could be assessed using specific markers in the subgranular zone of the dentate gyrus of the offspring hippocampus at PND 0, 7 and 21.

Alterations of neuron formation in the offspring of diabetic females could then be further correlated with possible changes in synapse formation.

We focussed our work in investigating hippocampal molecular changes at PND21 and looked for changes in synaptic proteins as synapsin-1, synaptophisin, PSD95 and KIF1A as indicating measures of altered synaptogenesis and did not found changes between CTRL and STZ offspring. It could be relevant to look at earlier timepoints, namely PND0 and PND7 since after birth the brain still undergoes extensive development. Maternal diabetes may alter synaptogenesis in earlier timepoints and due to the brain plasticity this changes became no longer observable at a later timepoint (ongoing work).

Additionally and in order to complement the western blot analysis of proteins related with synaptogenesis it could be a more reliable to assess synaptogenesis impairment to evaluate actual synaptic structures. We could perform Golgi staining and evaluate dendritic spine formation in order to determine if maternal diabetes would lead to an altered number of dendritic spines formation, or by immunohistochemistry label pre- and postsynaptic terminals and evaluate co-localization to determine if there would be less synapses. Ultrastructural imaging using transmission electron microscopy (TEM) could also be a tool to be considered for a morphological analysis of synaptogenesis as it enables a definitive

visualization of pre- and post-synaptic structures and allowing its characterisation (Bradford 2015).

If changes are found in the number of synapses, it could be related to changes in synaptic pruning. To discern if the synaptic number alteration could be due to synaptic pruning we could look at microglial cell activation and morphology in the hippocampus as these cells are key regulator of synaptic pruning. CX3C chemokine receptor 1 (CX3CR1) is a fractalkine receptor that can be found exclusively on microglia within the healthy brain. The fractalkine signaling plays a critical role on the regulation of several microglial properties as their migration, dynamic surveillance of the brain. During development it plays a role in neuron survival maturation, activity and plasticity and developing of mature synapse and synaptic pruning (Paolicelli et al. 2014). Therefore we could look to CX3CR1 expression and protein levels in hippocampal microglia. Additionally we could also look toward microglia morphology, performing immunostaining with a microglial cell marker, as Iba-1, and 3D reconstruction of microglial cells using the Neurolucida software (Caetano et al. 2016).

- Does maternal diabetes affect offspring hippocampal neurotransmission?

Even though we could not observe differences in VGluT-1 protein levels in the hippocampus of STZ offspring comparing with CTRL ones, as well as no changes in synaptic proteins that also play a role in exocytosis/neurotransmitter release, it would be important to evaluate the release of GABA and glutamate using a superfusion system and by using magnetic resonance imaging, asses GABA and glutamate levels in offspring hippocampus by *in vivo* Neurospectroscopy.

- Does maternal diabetes affect offspring hippocampal connectivity and function?

We observed a possible differential expression of AMPA receptor subunit at the hippocampal synaptic level that deserves to be further explored. Since AMPA receptors are either inserted or removed from synapses, resulting in the potentiation or depression of synaptic transmission, and subunit composition is responsible for differences in the functional activity of AMPA receptors, it would be important to perform electrophysiology recording of induced LTP in offspring hippocampal slices.

Also, offspring hippocampal integrity and connectivity could be evaluated by *in vivo* magnetic resonance imaging.

- Does maternal diabetes lead to changes in offspring memory and cognition?

In the present study, we only used the NOR test to evaluate short-term memory but we consider that, more than performing the NOR in a new set of animals to allow for a behavioural analysis with gender distinction, it would be interesting to perform other cognitive behavioural tests. For instances, the Morris Water Maze and Radial Maze, widely accepted spatial learning paradigms, that allow the attainment of several behavioral indexes of contextual/spatial habituation and cue driven navigation (Quillfeldt 2006).

Although it was not the main objective of this project, since in this study we assessed anxious-like behavior as a complementary information for cognitive performance, the results obtained may also open new avenues to explore the impact of maternal diabetes on offspring psychiatric health, since studies show that children of diabetic woman have more risk to suffer from autism and schizofrenia.

Additionally, in the context of all analysed parameters it would be of the upmost importance to evaluate if the alterations observed in the offspring of diabetic dams could be prevented upon administration of subcutaneous insulin implants for glycaemia control during pregnancy (Luippold et al. 2016).

Chapter 8

8. References

8. References

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Chapter 9

9. Supplementary Data

9. Supplementary Data

Table 4 – **Summary of offspring bodyweight from PND0-PND21. Results described in Chapter 4.1.1.** *Significance relative to CTRL male; #Significance relative to CTRL female.

	CTRL ♂			STZ ♂			CTRL ♀			STZ ♀		
	Mean (g)	SEM (g)	N	Mean (g)	SEM (g)	N	Mean (g)	SEM (g)	N	Mean (g)	SEM (g)	N
PND0	6,2	0.1	27	6.1	0.1	25	5.7	0.2	18	5.6	0.1	13
PND7	13.6	0.3	27	12.3$^{p=0.081}$	0.7	24	13.0	0.4	18	11.6	0.9	13
PND14	25.8	0.6	27	20.8**	1.4	24	24.2	0.7	18	20.2$^{\#}$	2.0	13
PND21	39.7	0.9	27	30.1***	2.2	24	37.5	0.9	18	29.3$^{\#\#}$	3.2	13

Table 5 –**Summary of offspring glycaemia from PND0-PND21. Results described in Chapter 4.1.1.** *Significance relative to CTRL male; #Significance relative to CTRL female.

	CTRL ♂			STZ ♂			CTRL ♀			STZ ♀		
	Mean (mg/dL)	SEM (mg/dL)	N	Mean (mg/dL)	SEM (mg/dL)	N	Mean (mg/dL)	SEM (mg/dL)	N	Mean (mg/dL)	SEM (mg/dL)	N
PND0	77.9	1.9	14	89.2*	4.6	9	82.6	3.1	15	94.5	10.0	6
PND7	130.5	4.0	10	145.3$^{p=0.085}$	3.9	3	130.3	2.1	15	142.3#	0.3	3
PND21	134.5	2.2	27	126.6$^{p=0.060}$	3.6	23	132.1	2.7	18	124.3	5.3	12

Table 6 – **Summary of offspring plasma insulin levels from PND0-PND21. Results described in Chapter 4.1.1.** *Significance relative to CTRL male; #Significance relative to CTRL female.

	CTRL ♂			STZ ♂			CTRL ♀			STZ ♀		
	Mean (µg/L)	SEM (µg/L)	N	Mean (µg/L)	SEM (µg/L)	N	Mean (µg/L)	SEM (µg/L)	N	Mean (µg/L)	SEM (µg/L)	N
PND0	0.197	0.023	3	0.166	0.031	3	0.230	0.017	4	0.179$^{p=0.073}$	0.016	4
PND7	0.139	0.004	3	0.172	0.019	3	0.151	0.006	4	0.153	0.006	4
PND21	0.141	0.006	3	0.156	0.009	3	0.159	0.007	3	0.145	0.002	3

Table 7 – **Summary of time (s) taken for pups (PND5-PND10) placed on their back to return to its four limbs (Surface Righting Reflex). Results described in Chapter 4.2.1.** *Significance relative to CTRL male; #Significance relative to CTRL female.

	CTRL ♂			*STZ ♂*			*CTRL ♀*			*STZ ♀*		
	Mean (s)	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*
PND5	1.2	0.1	26	1.4$^{p=0.078}$	0.1	24	1.1	0.1	17	1.5$^{p=0.053}$	0.2	12
PND6	1.0	0.0	26	1.1$^{p=0.059}$	0.1	23	1.0	0.0	18	1.1$^{p=0.096}$	0.1	12
PND7	1.1	0.1	26	1.0	0.0	22	1.0	0.0	17	1.3	0.3	12
PND8	1.0	0.0	26	1.4**	0.1	24	1.0	0.0	16	1.5$^{\#\#}$	0.1	13
PND9	1.0	0.0	12	1.1	0.1	23	1.0	0.0	10	1.2	0.1	12
PND10	1.2	0.1	13	1.2	0.1	19	1.2	0.1	11	1.4	0.2	10

Table 8 – **Summary of latency (s) for pups (PND5-PND14), to reverse orientation and face upwards on a 35-degree inclined platform (Negative Geotaxis Reaction). Results described in Chapter 4.2.1.** *Significance relative to CTRL male; #Significance relative to CTRL female.

	CTRL ♂			*STZ ♂*			*CTRL ♀*			*STZ ♀*		
	Mean (s)	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*
PND5	26.6	1.2	27	24.1	1.9	24	26.8	1.5	18	23.9	2.3	13
PND6	21.6	1.6	27	24.0	1.8	24	25.2	1.8	18	24.2	2.5	13
PND7	18.0	1.8	27	19.2	1.8	24	18.6	2.3	18	20.0	3.1	13
PND8	14.3	1.8	27	19.3*	1.8	24	17.5	2.2	18	23.7$^{p=0.075}$	2.5	13
PND9	7.5	0.8	12	12.6**	1.0	24	8.9	1.3	11	13.7$^{p=0.097}$	2.3	13
PND10	8.1	0.9	13	11.3*	1.1	18	9.2	1.4	11	9.8	2.4	10
PND11	7.8	0.7	27	12.5**	1.5	19	11.6	1.5	18	14.9	2.5	10
PND12	9.5	1.0	27	11.0	1.4	24	11.2	1.4	17	8.6	1.2	13
PND13	6.9	0.6	26	12.1***	1.3	24	5.8	0.5	17	13.5$^{\#\#\#}$	2.3	13
PND14	6.5	0.5	25	10.4*	1.5	24	6.6	0.6	18	12.0$^{\#}$	2.5	13

Table 9 – **Summary of time (s) taken for pups (PND5-PND10) placed hanging over an edge to retraction from it (Cliff Aversion). Results described in Chapter 4.2.1.** *Significance relative to CTRL male; #Significance relative to CTRL female.

	CTRL ♂			*STZ ♂*			*CTRL ♀*			*STZ ♀*		
	Mean (s)	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*
PND5	17.8	3.8	8	20.3	2.7	19	13.3	3.3	6	20.2	4.0	10
PND6	8.4	2.7	13	16.8*	2.7	19	18.4	3.5	11	19.4	3.9	10
PND7	9.0	2.7	13	13.0	2.5	19	11.5	3.6	11	15.6	4.0	10
PND8	4.4	0.6	13	11.3*	2.5	18	4.7	0.6	10	14.4*	4.3	10
PND9	2.9	0.2	8	7.2*	1.2	19	2.8	0.3	6	6.1$^{p=0.090}$	1.4	9
PND10	2.7	0.2	13	4.9*	0.8	18	4.2	0.6	10	10.0	3.5	10

Table 10 – **Summary of time (s) pups (PND10-PND14) spent hanging on the wire (Wire Suspension). Results described in Chapter 4.2.2.** *Significance relative to CTRL male; #Significance relative to CTRL female.

	CTRL ♂			*STZ ♂*			*CTRL ♀*			*STZ ♀*		
	Mean (s)	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*
PND10	5.2	0.8	13	5.5	0.9	19	7.3	0.8	11	4.9	1.3	10
PND11	5.8	0.8	13	6.4	0.9	19	7.3	1.0	11	6.1	1.3	10
PND12	5.7	0.6	27	5.6	0.8	19	5.2	0.8	18	5.6	1.5	10
PND13	7.8	0.5	26	6.9	0.7	24	8.8	0.5	16	6.5$^{\#}$	1.0	13
PND14	9.8	0.2	25	6.8***	0.7	24	8.2	0.7	18	7.2	0.9	13

Table 11 – **Summary of time (s) taken for pups (PND5-PND14) to fully exit the arena with all four limbs (Locomotion). Results described in Chapter 4.2.2.** *Significance relative to CTRL male; #Significance relative to CTRL female.

	CTRL ♂			*STZ ♂*			*CTRL ♀*			*STZ ♀*		
	Mean (s)	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*
PND5	30.0	0.0	26	29.8	0.2	23	30.0	0.0	17	30.0	0.0	13
PND6	26.8	1.2	27	30.0*	0.0	23	30.0	0.0	17	30.0	0.0	12
PND7	26.8	1.1	27	28.2	0.8	23	28.9	0.8	17	30.0	0.0	13
PND8	25.4	1.2	27	28.4$^{p=0.072}$	0.9	23	29.5	0.4	17	30.0	0.0	13
PND9	24.9	2.1	13	29.7*	0.2	18	22.0	2.8	11	29.0$^{\#}$	0.7	10
PND10	22.4	2.3	13	25.3	1.9	19	29.5	0.3	10	29.1	0.8	9
PND11	22.9	1.3	27	26.9$^{p=0.051}$	1.4	19	19.3	1.5	18	28.0$^{\#\#\#}$	1.0	10
PND12	19.3	1.5	27	25.2**	1.3	24	19.8	1.9	18	26.5$^{\#}$	1.6	13
PND13	16.9	1.5	26	24.3**	1.7	24	16.5	2.3	17	28.2$^{\#\#\#}$	1.2	12
PND14	15.0	1.8	27	14.1	1.6	24	12.1	2.0	18	19.4$^{\#}$	2.5	13

Table 12 – **Summary of latency (s) for pups (PND5-PND15) to transpose the apparatus home bedding goal mark with both snout and forelimbs (Nest Seeking). Results described in Chapter 4.2.3.** *Significance relative to CTRL male; #Significance relative to CTRL female.

	CTRL ♂			*STZ ♂*			*CTRL ♀*			*STZ ♀*		
	Mean (s)	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*	*Mean (s)*	*SEM (s)*	*N*
PND5	120.0	0.0	7	104.6	6.2	19	120.0	0.0	5	120.0	0.0	9
PND6	50.0	10.3	13	83.8*	9.3	19	67.8	16.2	11	87.1	12.1	10
PND7	25.2	6.7	12	30.8	6.5	19	63.7	11.7	11	65.4	13.5	10
PND8	41.8	9.6	13	25.9	4.4	18	23.9	5.5	10	52.0$^{p=0.078}$	13.9	10
PND9	31.6	10.1	13	37.8	8.2	19	23.9	7.5	11	44.3	13.6	10
PND10	12.4	4.9	12	26.0	5.8	19	17.2	6.0	10	15.5	1.8	9
PND11	13.6	4.8	12	11.8	1.6	18	21.9	7.8	11	26.3	6.3	10
PND12	13.6	4.2	26	16.3	4.1	19	4.5	0.5	17	23.5$^{\#\#}$	7.7	10
PND13	12.3	3.5	27	13.1	4.2	24	22.2	6.5	17	5.9$^{\#}$	0.8	12
PND14	2.7	0.2	25	5.9***	0.8	23	15.7	5.9	18	6.5	1.3	13
PND15	2.4	0.2	27	12.8**	4.0	24	2.9	0.2	18	8.4$^{\#\#\#}$	1.6	12

Table 13 – **Summary of percentage of pups with eyes open per day (%) from PND12-PND17. Results described in Chapter 4.2.4.** *Significance relative to CTRL male; #Significance relative to CTRL female.

	CTRL ♂			*STZ ♂*			*CTRL ♀*			*STZ ♀*		
	Mean (%)	*SEM (%)*	*N*	*Mean (%)*	*SEM (%)*	*N*	*Mean (%)*	*SEM (%)*	*N*	*Mean (%)*	*SEM (%)*	*N*
PND12	0.0	0.0	27	0.0	0.0	24	0.0	0.0	18	0.0	0.0	13
PND13	0.0	0.0	27	0.0	0.0	24	0.0	0.0	18	0.0	0.0	13
PND14	7.4	5.1	27	12.5	6.9	24	0.0	0.0	17	15.4	10.4	13
PND15	59.5	9.6	27	25.0*	9.0	24	72.2	10.9	18	38.5$^{p=0.063}$	14.0	13
PND16	100.0	0.0	18	36.8****	11.4	19	100.0	0.0	18	61.5$^{\#\#}$	14.0	13
PND17	100.0	0.0	22	62.5***	10.1	24	100.0	0.0	18	69.2$^{\#}$	13.3	13

Table 14 – **Summary of percentage of pups with eyes open per day (%) from PND12-PND17. Results described in Chapter 4.2.4.** *Significance relative to CTRL male; #Significance relative to CTRL female.

	CTRL ♂			*STZ ♂*			*CTRL ♀*			*STZ ♀*		
	Mean (%)	*SEM (%)*	*N*	*Mean (%)*	*SEM (%)*	*N*	*Mean (%)*	*SEM (%)*	*N*	*Mean (%)*	*SEM (%)*	*N*
PND11	0.0	0.0	8	0.0	0.0	19	0.0	0.0	6	0.0	0.0	10
PND12	0.0	0.0	8	0.0	0.0	19	0.0	0.0	6	0.0	0.0	10
PND13	0.0	0.0	8	0.0	0.0	18	0.0	0.0	5	0.0	0.0	10
PND14	100.0	0.0	8	52.6*	11.8	19	100.0	0.0	6	60.0$^{p=0.082}$	16.3	10