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Diabetic encephalopathy and neuroactive steroids: observations in female rats

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ABBREVIATIONS

17β-HSD: 17β-hydroxysteroid dehydrogenase

3α-HSOR: 3α-hydroxysteroid oxidoreductase

3β-HSD: 3β-hydroxysteroid dehydrogenase

3β-HSOR: 3b-hydroxysteroid oxidoreductase

5α-R: 5α-reductase

ALLO: allopregnanolone

ANS: autonomic nervous system

AR: androgen receptor

ARO: aromatase

BB-DP: Bio-Breeding diabetes-prone

BB-DR: Bio-Breeding diabetes-resistant

BB: Bio-Breeding

BDNF: brain-derived neurotrophic factor

BMI: body mass index

CB: cerebellum

Cldn-1: claudine-1

CNS: central nervous system

CRH: corticotropin releasing hormone

CTX: cerebral cortex

DE: diabetic encephalopathy

DHEA: dehydroepiandrosterone

DHP: dihydroprogesterone

DM: diabetes mellitus

ENS: enteric nervous system

ER: estrogen receptor

ETS: electron transport system

GABA: gamma amino-butyric acid

GC: glucocorticoids

GLUT-2: glucose transporter-2

HLA: human leucocyte antigen

HPA: hypothalamic–pituitary-adrenal

i.p.: intraperitoneal

IL: interleukin

IMM: inner mitochondrial membrane

ISOALLO: isoallopregnanolone

IVC: individually ventilated cage

MnSOD: manganese superoxide dismutase

mPRs: membrane progesterone receptors

Muc-2: mucine-2

NDDs: neurodegenerative disorders

NF-kB: nuclear fator-kappaB

NMDA: N-Methyl-D-aspartate

NO: nitric oxide

NOD: non-obese diabetic

NOR: Novel object recognition

P450scc or CYP11A1: cytochrome P450 side chain cleavage

PNS: peripheral nervous system

PR: progesterone receptor

PREG: pregnenolone

PROG: progesterone

PROG: progesterone

PXR: pregnane X receptor

RNS: reactive nitrogen species

ROS: reactive oxygen species

RR: relative risk

SC: spinal cord

SCFA: short-chain fatty acids

SNAP-25: synaptosomal-associated protein

StAR: steroidogenic acute regulatory protein

STZ: streptozotocin

T1DM: Type 1 diabetes mellitus

TCA: tricarboxylic acid cycle

THP: tetrahydroprogesterone

TNF: tumor necrosis factor

TSPO: translocator protein 18KDa

UCPs: uncoupling proteins

VDAC: voltage-dependent anion channel protein

ZO-1: zonuline-1

ABSTRACT

Diabetes mellitus (DM), a chronic metabolic disorder, may induce neurophysiological and structural changes in the central nervous system (i.e., diabetic encephalopathy). Diabetic encephalopathy (DE) is one of the most common and severe chronic complications of DM, characterized by impaired cognitive and memory functions, and structural, neurochemical, and electrophysiological abnormalities. These alterations are associated with decline in cognitive processes, increased risk of cerebrovascular and Alzheimer's disease, dementia, and psychiatric disorders.

Memory and cognitive impairments are associated with hippocampal dysfunction, but the mechanisms behind these alterations are not fully known. Based on data collected in literature, the development of cognitive deficit seems to be the result of the concomitant presence of different processes such as mitochondrial dysfunction, oxidative stress, neuroinflammation and aberrant synaptogenesis in several brain areas of male rat model of type 1 DM (T1DM). Another important factor involved in the development of DE are steroid molecules because it is widely known that these molecules are regulators of the nervous function. Indeed, our previous data have shown that levels of many neuroactive steroids (i.e., steroids affecting the nervous system functionality) are decreased by three months (long-term) of DM but also by one month (short-term) in male streptozotocin (STZ)-induced rats.

Moreover, DE presents differences, between two sexes, in term of incidence, progression and severity of the pathology. However, despite DE shows many sex-dimorphic features, no observations in female rats have been so far reported in literature.

On this basis, in the present study, firstly, we have explored the impact of one month of diabetes on memory abilities by Novel Object Recognition (NOR) test, on mitochondrial functionality, oxidative stress, synaptogenesis and neuroactive steroids levels in hippocampus and cerebral cortex of female STZ-rats in order to understand whether short-term DM affects these parameters also in females and whether possible changes are different in the two sexes.

Data reported in this thesis indicate that short term of T1DM is able to significantly decrease memory abilities. This result is associated with aberrant synaptogenesis (i.e., decrease in the levels of synaptophysin, synapsin and syntaxin) and neuroinflammation (i.e., increased levels of IL-1β and IL-6) in hippocampus of female STZ-rats. Oxidative stress and mitochondrial functionality are not affected in this brain area, possibly due to protective effect of dihydroprogesterone (DHP) and allopregnanolone (ALLO), whose hippocampal levels are significantly increased. These effects are specific for hippocampus since the cerebral cortex does not present a similar increase in steroid levels and consequently shows increased neuroinflammation and oxidative stress possibly due to mitochondria dysfunction.

T1DM induced not only a diabetic encephalopathy but also dysbiosis. On this basis, we focused our attention on the possible role of gut microbiota on the development of memory deficits in female STZ-rats in the contest of gut-brain axis. In particular, we explored, whether DM can affect the composition of gut microbiota, the levels of steroids in colon and gut permeability markers. In addition, we also evaluated whether correlations may occur among these parameters in female STZ-rats.
Results obtained demonstrated that T1DM alters gut β-, but not α-diversity. The pathology is also associated with a significant decrease in pregnenolone (PREG) and increase in ALLO levels in the colon of female STZ-rats. In addition, T1DM altered gut permeability (i.e., decreased of zonulin-1 and claudin-1). Interestingly, we reported a significant correlation of PREG with *Blautia,* claudin-1 and the NOR index and of ALLO with *Parasutterella, Gammaproteobacteria* and claudin-1.

Altogether, data obtained in this thesis suggest a pivotal role of neuroactive steroids and gut steroids in the development of memory disfunctions. In the central nervous system, increased levels of DHP and ALLO seem to be an attempt to counteract the negative effects of T1DM particularly in hippocampus. In periphery, the positive correlation between decreased level of PREG in colon and

behavioural results, support the hypothesis that this steroid has an important rol in the development of cognitive deficit observed in the CNS across the gut-brain-axis.

RIASSUNTO

Il diabete mellito (DM) è una patologia metabolica cronica che può provocare alterazioni neurofisiologiche e cambiamenti strutturali nel sistema nervoso centrale (SNC). Tale condizione è nota come encefalopatia diabetica (ED). L'encefalopatia diabetica è una delle complicanze a lungo termine più comuni e gravi di questa patologia, caratterizzata da alterazioni cognitive e della memoria. Essa induce cambiamenti di tipo strutturale, neurochimico ed elettrofisiologico a livello nervoso. In generale questi cambiamenti sono associati ad un progressivo declino cognitivo, aumentato rischio di sviluppare malattie cerebrovascolari ma anche Alzheimer, demenza e disordini psichiatrici.

Le alterazioni delle funzioni cognitive e della memoria sono associate a disfunzioni a livello ippocampale ma, i meccanismi sottesi a tali cambiamenti, non sono ancora completamente noti.

I dati presenti in letteratura suggeriscono che, lo sviluppo dei deficit cognitivi sia il risultato della concomitante presenza di diversi meccanismi biomolecolari quali stress ossidativo, alterazioni delle normali funzioni mitocondriali, neuroinfiammazione e sinaptogenesi aberrante evidenziate in differenti aree cerebrali, quali, ad esempio, ippocampo e corteccia cerebrale, in modelli sperimentali di ratto maschio di diabete di tipo 1. Un ruolo cruciale nello sviluppo dell'ED è ricoperto anche dalle molecole steroidee in quanto è ampiamente noto che esse sono importanti regolatori delle funzioni nervose, tra cui i processi cognitivi e di memoria. Infatti, diversi nostri lavori hanno dimostrato come il diabete in un modello di ratto maschio di DM di tipo 1, streptozotocina (STZ)-indotto, sia dopo tre mesi, DM a lungo termine, che dopo un mese, DM a breve termine, riduce in modo significativo i livelli di importanti steroidi neuroattivi, ovvero steroidi in grado di influenzare le funzionalità nervose.

Inoltre, l'encefalopatia diabetica, come la neuropatia diabetica, presenta differenze tra i due sessi in termini di incidenza, progressione e severità della patologia. Tuttavia, nonostante sia ampiamente noto che l'ED presenti queste caratteristiche sessualmente dimorfiche, ad oggi non sono riportate in letteratura osservazioni sperimentali in modelli femminili di DM.

Sulla base di quanto detto, in questo studio, abbiamo deciso di esplorare l'impatto di un mese di diabete in un modello STZ- indotto di ratto femmina. In particolare, abbiamo valutato le abilità di memoria ricorrendo al test comportamentale *Novel Object Recognition (NOR)*, lo stress ossidativo, la funzionalità mitocondriale e la sinaptogenesi grazie a diverse tecniche di biologia molecolare e, infine, i livelli degli steroidi neuroattivi mediante la spettrometria di massa. Le analisi sono state condotte nella corteccia cerebrale e nell'ippocampo, per cercare di comprendere come un mese di DM possa influenzare questi parametri anche nelle femmine e se queste potenziali alterazioni siano o meno differenti tra i due sessi.

I dati ottenuti in questa tesi indicano che il DM a breve termine è in grado di alterare significativamente le abilità di memoria delle ratte diabetiche. Ciò è associato ad un'alterata sinaptogenesi caratterizzata da una riduzione significativa dei livelli di tre importanti proteine sinaptiche, la sinaptofisina, la sinapsina e la sintaxina, e ad un maggiore rilascio di interleuchine, IL-6 e IL-1β con conseguente neuroinfiammazione a livello ippocampale del gruppo STZ rispetto al gruppo controllo. Tuttavia, in questa area cerebrale, non abbiamo riscontrato un'alterazione della funzionalità mitocondriale e dello stress ossidativo. Questo potrebbe essere dovuto all'effetto protettivo del diidroprogesterone (DHP) e dell'allopregnanolone (ALLO), i cui livelli ippocampali sono aumentati significativamente. Complessivamente questi effetti sembrano essere specifici per l'ippocampo in quanto nella corteccia cerebrale, nella quale sono state eseguite le medesime analisi, non è stato evidenziato un aumento di tali steroidi neuroattivi e di conseguenza, come ipotizzato, abbiamo osservato un aumentata neuroinfiammazione ma anche stress ossidativo possibilmente dovuto ad un'alterata funzionalità mitocondriale, evidenziata mediante l'analisi degli OXPHOS.

Il DM di tipo 1 oltre ad indurre nel lungo termine encefalopatia diabetica, è in grado di provocare anche disbiosi. Su questa base abbiamo deciso di focalizzare la nostra attenzione anche sul possibile ruolo del microbiota intestinale nello sviluppo dei deficit di memoria nel medesimo modello femminile di DM di tipo 1

nel contesto dell'asse intestino-cervello. Nel dettaglio, abbiamo esplorato come il DM possa alterare la composizione del microbiota intestinale, i livelli degli steroidi nel colon e i marker della permeabilità intestinale nelle ratte diabetiche rispetto al gruppo controllo. Inoltre, è stato interessante approfondire quali possibili relazioni intercorressero tra i parametri valutati nelle ratte diabetiche.

I risultati che abbiamo ottenuto dimostrano che il DM di tipo 1 altera la β-diversità intestinale ma non la α-diversità. La condizione diabetica è inoltre associata ad una riduzione significativa dei livelli di pregnenolone (PREG) e un aumento significativo di un altro importante steroide, l'allopregnanolone (ALLO) nel colon delle ratte STZ. Un altro interessante risultato è stato evidenziato dall'analisi di importanti markers della permeabilità intestinale, la zonulina-1 e la claudina-1, che sono risultate significativamente ridotte. Inoltre, dalle analisi delle possibili correlazioni tra questi parametri abbiamo individuato una significativa correlazione tra il PREG e i *Blautia,* la claudina-1 e il NOR ed infine l'ALLO e *Parasutterella, Gammaproteobacteria* e la claudina-1.

In conclusione, i dati ottenuti in questa tesi suggeriscono un ruolo cruciale degli steroidi neuroattivi e degli steroidi intestinali nello sviluppo dei deficit di memoria. Nel sistema nervoso centrale, l'aumento dei livelli di DHP e ALLO sembra essere un tentativo di contrastare gli effetti negativi del DM, soprattutto a livello ippocampale. In periferia, invece, la correlazione positiva tra i livelli ridotti di PREG nel colon e i risultati del test comportamentale, supportano l'ipotesi che il PREG sia importante nello sviluppo dei deficit cognitivi osservati nel SNC attraverso l'asse intestino-cervello.

INTRODUCTION

Diabetes mellitus: general concepts

Diabetes mellitus (DM) is a general term used to indicate heterogeneous disturbances of metabolism characterized by high blood glucose levels (hyperglycemia), with changes in glucose, lipid, and protein metabolism. This pathology has a multiple etiology due to the interaction of environmental and genetic factors that induce a deficit in the secretion of hypoglycemic hormone, insulin and/ or a reduction in its biological activity.

Diabetes mellitus, a disease of endocrine system, is one of the most common and fastest growing pathologies worldwide, projected to affect 693 million adults by 2045 (Cho et al., 2018), a >50% increase from 2017. Patients affected by diabetes have increased a risk to develop different health problems affecting important anatomic districts. Particularity, vascular complications both the macrovascular system (cardiovascular disease) and microvascular system (diabetic kidney disease, diabetic retinopathy, encephalopathy, and neuropathy) are the primary cause of morbidity and mortality in patients with diabetes (Morrish et al., 2001).

The classic classification of DM provides an early-onset autoimmune form, type 1 diabetes mellitus (T1DM), and a late-onset non-autoimmune form, T2DM. Moreover, there are other clinically recognizable subtypes such as gestational diabetes, monogenic diabetes (for example maturity-onset diabetes of the young or neonatal diabetes) and a late-onset autoimmune form, known as *latent autoimmune diabetes* in the adult. T1DM corresponds to 5-10% of people with diabetes and T2DM affects the remainder, i.e., about 90% of those with the disease. Gestational diabetes instead affects about 5% to 6% of pregnant women and in most instances is an early form of T2DM (data from "*Diabetes Public Health Resources: diabetes statistics from NIDDK, Center for Disease Control, and the National Diabetes Information Clearinghouse; 2004).*

T1DM is a chronic, immune-mediated disease, characterized by the destruction of insulin-producing β cells in the pancreas and resultant hyperglycemia. Recently data show that T1DM incidence has increased 3-4% over the past three decades. The production of β cells autoantibodies could be induced after an exposure of

genetically susceptible individual to a presumed environmental factor which triggers a loss of immune regulation (Atkinson et al., 2014). The disruption of these pancreatic cells leads to a decrease in insulin secretion, development of hyperglycemia and then clinical type 1 diabetes.

Particularly, the genes responsible for this pathology are carried on the DQ of the short arm of the chromosome 6. Genes in this area are known as the Major Histocompatibility System and control the immune system. The immune process occurs in individuals with a genetic hereditary predisposition associated with HLA (human leucocyte antigen) class II molecules, which are responsible to present the antigen to the T helper cells and then start the immune response. Specifically, individuals with certain haplotypes of HLA (DR3 and DR4) have a higher risk of developing T1DM than to the general population (Noble et al., 2008). When some of these gene malfunction or are abnormal, they could impair the ability of immune system to recognize itself triggering autoimmune diseases such as T1DM. As regard the environmental factors that trigger the destruction of β cells, they are not yet fully identified. Some viruses, such as coxsackie virus and rubella virus, have been identified in some patients (Andreoletti et al., 1997). Also, cow's milk proteins, in particular β–casein, have an aminoacidic sequence very similar to one present in glucose transporter-2 (GLUT-2). It has hypothesized that these sequences caused in children an immunological reaction, due to a mechanism of molecular mimicry (Pozzilli, 1999; Monetini et al., 2002).

Patients affected by DM have increased a risk to develop several numbers of health problems. Indeed, diabetic complications can be divided into "acute complications" due to metabolic changes that, if not promptly and properly treated, may quickly cause the death of patients, and "long-term complications" caused by the state of chronic hyperglycemia that causes functional and structural alterations of specific organs and tissues. Acute metabolic complications associated with mortality include diabetic ketoacidosis caused by high blood glucose levels (hyperglycemia) and diabetic coma as the result of low blood glucose (hypoglycemia).

Long term complications appear about 10-20 years after the onset of diabetes, and they are the main cause of morbidity and mortality associated with DM. Depending on the severity, pharmacological control and duration of the DM, there is an increase in their incidence. These complications are due at least in part to chronic high blood glucose levels which cause important metabolic imbalances and lead to damage of blood vessels. In diabetes, the resulting complications are grouped under "macrovascular disease" (due to damage to the arteries) and "microvascular disease" (due to damage to small blood vessels). The first affect the coronary, cerebral and renal arteries, and lower limbs (diabetic foot), while the latter affect the small vessels, capillaries, and arterioles. Microvascular complications include eye disease or "retinopathy," kidney disease termed "nephropathy," and neural damage or "neuropathy". Diabetic peripheral neuropathy, as referred to impairment of the peripheral nervous system, affects more than 50% of diabetic patients. This condition presents a sex-dimorphic features in term of incidence and progression. Indeed, diabetic neuropathy is more frequent in men than in woman (Basit et al., 2004; Booya et al., 2005). Moreover, males develop neuropathy earlier than females (Aaberg et al., 2008).

In addition, diabetic condition also causes non-vascular complications which mainly affect the oral cavity and the gastrointestinal tract, the urogenital system, causing urinary infections and sexual dysfunction, the eye (cataracts) and the skin characterized by diabetic dermopathy, ulcerations and modification in the mechanism of scarring. However, even the central nervous system (CNS) is not spared from the deleterious effects of DM, since several studies have described neuropsychological and neurobehavioral changes in diabetic subjects, suggesting that diabetic encephalopathy should be recognized as an important complication of this complex metabolic disorder.

Diabetic encephalopathy

Diabetic encephalopathy (DE) is one of the most severe complications of diabetes, characterized by impaired cognitive and memory functions, and electrophysiological, neurochemical, and structural abnormalities (Cai et al., 2011). These alterations are associated with cognitive deficits and increase risk of dementia, stroke, cerebrovascular and Alzheimer’s disease and psychiatric disorders, such as depression, and eating disorders (Gispen and Biessels, 2000; Biessels et al., 2002, Jacobson et al., 2002; Biessels et al., 2008; Kodl and Seaquist, 2008). DE represents the long-term neurological complication of diabetes (Biessels and Gispen, 2005) and its prevalence is 40% in long standing and poorly controlled diabetes (Dejgaard et al., 1991).

It is interesting to underline that incidence, progression, and severity of diabetic encephalopathy as well as diabetic neuropathy are different in two sexes. In contrast to what was previously mentioned about the sexual known differences of peripheral neuropathy, it is unknown whether there are also sex differences in cognitive function related to diabetes. As regard diabetic encephalopathy, diabetes is a risk factor for dementia, but whether the association is similar in women and men remains poorly explored. *Chatterjee and collaborators* performed a meta-analysis of data collected from clinical study to estimate the sex-specific relationship between women and men with T2DM with incident of dementia (Chatterjee et al., 2016). From these meta-analyses, diabetes was associated with a 60% of dementia in both sexes (women: pooled relative risk (RR) 1.62; men: pooled RR: 1.58). The relative risks change when it considered DM associated with vascular and non-vascular dementia. For vascular dementia, but not for non-vascular dementia, the additional risk is greater in women. Real biological differences between women and men support the higher risk of diabetes-related vascular risk in women. For example, exposure to endogenous estradiol in women may also play a role. Indeed, *Adam et al*., performed a study among post-menopausal women and observed that higher levels of endogenous estradiol, especially in women with DM, conferred a greater risk of dementia

(Adam et al., 2007). Although fewer studies have examined the role of sex differences on cognitive outcomes in people with T1DM (Brands et al., 2005; van Duinkerken et al., 2012). Also, in patients with T1DM, it is important to consider the protective role of female hormones on cognitive impairments and on vascular risks and the implication of menopause on this loss of protection and long-term brain health.

In type 1 diabetes, cognitive dysfunction emerges early in the disease course (within 2 years of diagnosis). Age is also an important variable, and children's brains might be more susceptible to the effects of diabetes than adult's brains. Individuals who develop T1DM early in life (young than 7 years) have a higher risk of developing more severe cognitive deficits than are those who develop diabetes at an older age (Ryan, 2006). Moreover, T1DM has a specific effect on a subset of cognitive domains in adults, including attention, intelligence, cognitive flexibility, psychomotor speed, and visual perception (Brands et al., 2005). In addition, it has been shown that early onset of type 1 DM results in worse neuropsychological performance (Schoenle et al., 2002; Ryan et al., 1985; Ryan, 2006) and that males are more vulnerable than females (Schoenle et al., 2002; Fox et al., 2003). Interestingly, in adult with either type 1 or type 2 diabetes, imaging studies have demonstrated smaller hippocampi, as well as changes in the functional connectivity between regions of the brain (Bolo et al., 2011, Musen et al., 2012, Antenor-Dorsey et al., 2013, Lyoo et al., 2013).

As it is widely known, the hippocampus is a crucial part of the limbic system, which plays a pivotal role in memory formation, emotional, adaptive, and reproductive behaviors (Squire, 1992). This brain region is also particularly important to connect emotions and senses, such as smell and sound, to memories (Turgut and Turgut, 2011). The structural complexity of hippocampus renders this brain structure vulnerable to the many pathological conditions and metabolic disorders including DM (Biessels et al., 1996, Alvarez et al., 2009, Pamidi and Satheesha Nayak, 2012, Foghi and Ahmadpour, 2013). Indeed*, Li and collaborators* have reported hippocampal neuronal death in spontaneous rat model of T1DM, accompanied by

some functional cognitive alteration after a long period of diabetes (8 months) (Li et al., 2002c). Moreover, rats and mice, raised diabetic with a single i.p. injection of streptozotocin (STZ), shows a decreased hippocampal cell proliferation (Guilford et al., 2000, Saravia et al., 2004, Beauquis et al., 2006, Kang et al., 2006, Stranahan et al., 2008, Zhang et al., 2008, Balu et al., 2009, Revsin et al., 2009, Wang et al., 2009a, Wang et al., 2009b, Piazza et al., 2011).

In the recent years, increasing evidence of cognitive dysfunction as well as memory impairment caused by diabetes, has been obtained in animal models (Biessels et al., 1996, Li et al., 2002b). In STZ mice and rats, model of T1DM, neurobehavioral deficits have been detected with the Morris water maze test, which is a spatial memory assay. These animals displayed also impaired hippocampal long-term potentiation (Biessels et al., 1996). Numerous studies have shown that experimental diabetes has negative impacts and induce apoptosis in hippocampal neurons via multiple mechanisms. However, the pathogenesis of DE in patients affected by T1DM but also in animal model is not completely clear, but the mechanism behind these impairments caused by diabetic conditions is very complex and not fully known. Among the more established mechanisms we have the persistent inflammation caused by the release of numerous pro-inflammatory factors such as interleukin (IL)-6, IL-β, *tumor necrosis factor* (TNF)-α, and oxidative substances such as ROS and RNS (Gaspar et al., 2016). Indeed, several studies have shown swelling of synapses and fragmentation of neurofilaments within the filaments, alterations of myelin membranes (Hernandez-Fonseca et al., 2009) and its components, namely lipids and proteins (Pesaresi et al., 2010; Cermenati et al., 2017) and impairment of axonal transport (Baptista et al. 2013) as well as swelling of axons and dendrites (Zhou et al.2013).

Surely, important factors present in the diabetic condition like decreased secretion or insulin action, alteration of glucose homeostasis, increased glucocorticoid levels, metabolic impairment (Bang et al., 2018; Zhao et al., 2018) as well as oxidative stress (Masola et al., 2018; Minaz et al., 2018) inflammation

(Gault and Holscher, 2018; Rom et al., 2018) mitochondrial dysfunction and apoptosis (Zhang et al., 2018) have been proposed as the main causes of the development of cognitive deficits in DM. Indeed, it has been demonstrated that hyperglycemia causes oxidative stress in different brain regions and alters the activation of enzymes which are considered pivotal for normal CNS function (Liu et al., 2016). Moreover, *Zhu et al.* evidenced that inflammatory process was highly related to the pathogenesis of cognition deficits (Zhu et al., 2016).

Several lines of evidence suggested that inflammation was a crucial contributor to the initiation and progression of cognitions deficits (Chen et al., 2015). Under chronic condition of hyperglycaemia, endogenous TNF-α is overproduced in microvascular as well as in neural tissues, which may increase nerve damage, microvascular permeability, and diabetic neuropathy as well diabetic encephalopathy (Satoh et al., 2003). Indeed, in STZ -diabetic male rats, diabetes caused the upregulations of IL-1β, TNF-α and IL-6 contents in hippocampus supported additionally the view that the overexpression of inflammatory cytokines were related to diabetes-associated cognitive impairment (Ma et al., 2016; Zhu et al., 2016; Baluchnejadmojarad et al., 2017).

Moreover, rats and mice, raised diabetic with a single i.p. injection of STZ, shows a decrease hippocampal cell proliferation (Jackson-Guilford et al., 2000, Saravia et al., 2004, Beauquis et al., 2006, Stranahan et al., 2008, Zhang et al., 2008, Balu et al., 2009, Revsin et al., 2009, Wang et al., 2009a, Wang et al., 2009b,). Cognitive impairment associated with the hippocampal dysfunction underlying diabetic encephalopathy have also been documented in studies of diabetic animal models induced with STZ, a commonly used agent to induce rodent model of T1DM through destroying insulin producing cells (Chatzigeorgiou et al., 2009) and/or high-fat diet (HFD). Development of cognitive dysfunction in STZ-diabetic rats has been widely demonstrated employing passive avoidance, Y-maze (Nasri et al., 2012) and novel object discrimination (NOR) tasks (Baluchnejadmojarad et al., 2017).

Potential mechanisms involved in the development of cognitive impairments in diabetes mellitus.

Data collected from literature in experimental model of DM and from patients suggest that the development of cognitive deficits seems to be the result of the concomitant presence of different processes such as oxidative stress (Masola et al., 2018; Minaza et al., 2018; Elahi et al., 2016), impairment of mitochondrial functionality (Silva-Rodrigues et al., 2020; Cardoso et al., 2010, 2013), neuroinflammation (Elahi et al., 2016; Pei et al., 2018; Xu et al., 2021; Jawale et al., 2016; Baluchnejadmojarad et al., 2017) and aberrant synaptogenesis characterized by synaptic protein loss and synaptic structure impairments (Biessels et al., 1996; Xu et al., 2021; Kuhad et al., 2009; Gaspar et al., 2016).

i) ***Mitochondrial dysfunction and oxidative stress***

As mentioned above, one of the mechanisms involved in the development of diabetic encephalopathy is the impairment of mitochondrial functionality normally linked to an increased oxidative stress.

Mitochondria are symbiotic organelles that contain their own genetic material, the mt-DNA, which encodes essential subunits of the respiratory chain complex I, III, IV, and V. Although their most well-known function is cellular energy production and conservation, mitochondria are central to cell cycle regulation, are involved in programmed cell death, calcium signaling, and redox homeostasis and signaling. Indeed, these organelles are involved in the response to pathological conditions that cause stress to the energy metabolism, such as diabetes mellitus.

Mitochondrial bioenergetics system

The mitochondrial electron-transport system consists of several multi-polypeptide protein complexes (I-V) embedded in the inner mitochondrial membrane (IMM) that receive electrons from reducing coenzyme such as NADH and FADH2 (*Figure 1*). These electrons are transferred through a series of electron carriers in the

respiratory chain, where O_2 serving as the final electron acceptor and is reduced into H_2O. Each of the electron carriers represents a redox couple (i.e., species capable of existing in a reduced or oxidized state) with a reduction potential. The electron carriers in the respiratory chain are ordered so that the reduction potentials progressively increase (i.e., become more positive) from one redox couple to another. In three of these complexes (I, III and IV), the difference in reduction potential (i.e., release of energy), across successive redox couples, is sufficient to drive the translocation of protons from the matrix to the inner membrane space. The energy release creates a proton gradient across the inner membrane which is derived from both the electrical potential and the concentration difference across the membrane. The core of the chemiosmotic theory is that the electrochemical energy created by the generation of the proton motive force is sufficient to drive the synthesis of ATP as well as to flow back, through the ATP synthase complex, the protons into the matrix (Fisher-Wellman and Neufer, 2012). The transport of electrons through the respiratory chain occurs automatically. For the most part, electron flow and proton pumping are closely coupled. To activate respiration, fuel (i.e., NADH, FADH2) is added which automatically initiates electron flow, proton pumping, and O_2 consumption. However, to regulate this process on the outer surface of the inner membrane, a "back pressure" is created which begins to oppose the pumping of protons, thereby gradually slowing electron flow and O_2 consumption. Mitochondria should never reach a state of "static head" equilibrium, where the force driving the pumping of protons out the matrix is completely counter-balanced by the high membrane potential. This basal rate of proton conductance ensures that the membrane potential is almost less than maximum, "allowing" electron flow, proton pumping, and O2 consumption, to operate at an "idling" rate (known as state 2 respiration). When another flux of proton is created to flow back into the matrix (e.g., via ATP synthase catalyzing re-phosphorylation of ADP), the back pressure of the membrane potential is further reduced accordingly with the proton pumping and oxygen consumption increase (known as state 3 respiration).

Once all of the ATP is synthesized, the system slows back to the idling rate (known as state 4 respiration). The crucial point is that the mitochondrial respiratory system is a “primer engine”, which automatically adjusts to each change in the rate of proton re-entry into the matrix, a corresponding change in electron flow and O_2 consumption. The respiratory system operates is governed by energy demand, and not by energy supply. Proton conductance (via leak and ATP synthase) dictates the rate of electron flow and therefore the demand for reducing equivalents (e.g., NADH and FADH2) that, in turn, regulates the rate of substrate uptake and flux through catabolic pathways (Fisher-Wellman and Neufer, 2012).

To maintain the normal brain function, a large amount of ATP produced is required. Among all tissues of the body, brain is the most energy dependent. The energy required for the normal functioning of the brain is largely produced by normal activity of mitochondria. ATP production is associated with an electron transport system (ETS) where, the passage of electrons through diverse electron carriers is coupled with the transport of protons from the mitochondrial matrix into the inner membrane space, and thereafter these protons re-enter into the mitochondrial matrix for the generation of ATP through ATP synthase (Onyango et al., 2010, Su et al., 2010).

The product of the reduction potential and the reducing capacity (i.e., the concentration of the reduced species) of a redox couple is the definition of the redox state. Several of the redox couples within the electron-transport chain transfer single rather than two electrons and are therefore susceptible to leaking electrons directly to surrounding O_2 to form the free-radical superoxide (O_2^-). Under state 3 conditions, the redox state of the system is below the threshold at which electrons will loss O_2. However, at or near state 4, the respiratory system is in its most reduced state such that the rate at which O_2^- is produced is extremely sensitive to the redox state of the system, increasing exponentially with even small increases in membrane potential (Korshunov et al., 1997, Liu, 1997, 1999). Fortunately, O_2^- is rapidly converted by manganese superoxide dismutase (MnSOD or SOD2) to the two-electron non- radical hydrogen peroxide (H_2O_2). H_2O_2

in turn can be further reduced to H_2O in mitochondrial matrix by glutathione (GSH) or the thioredoxin/peroxiredoxin systems or can freely diffuse out of the mitochondrial where it again is buffered by GSH. Thus, in a resting cell, the mitochondrial respiratory system functions as redox pressure-gauge that senses and reflects cellular metabolic balance. When in positive balance, electron leak serves as release valve, accelerating mitochondrial O_2^- production and H_2O_2 emission (Fisher-Wellman and Neufer, 2012). Superoxide anions do not readily cross membranes (Fridovich, 1986), but may be transported by anion channels (Kontos et al., 1985). They rapidly react with NO, forming peroxynitrite (ONOO-), which is a mediator of neurodegeneration (Estevez et al., 1995, Schulz et al., 1995, Szabo, 1996), that may damage and kill cells by induction of lipid peroxidation and protein tyrosine nitration (Beckman, 1994, Beckman et al., 1994). Hydrogen peroxide easily penetrates lipid bilayers, acts as an oxidizing agent, and is relatively stable, although it is not a free radical. Moreover, this molecule helps modulate signaling system in the cell, such as kinase and phosphatase (Whisler et al., 1995, Denu and Tanner, 1998) and transcription of genes (Mattson and Camandola, 2001). Hydrogen peroxide is not toxic except in high concentration of hydroxyl radicals (O_2- + H_2O_2 -> OH + OH- + O_2), particularly in the presence of ferrous ions, which are present in the brain parenchyma (e.g., after trauma and intracerebral hemorrhage). Hydroxyl radicals are extremely reactive, and rapidly attack unsaturated fatty acids in membranes causing lipid peroxidation and the production of 4-hydroxynonenal that conjugates to membrane proteins, impairing their function (Keller et al., 1997a, Keller et al., 1997b). During homeostasis, the production of ROS is balanced by antioxidant system such as SOD, catalase, glutathione peroxidase and glutathione reductase, maintaining the levels of $O_2^{\bullet -}$ and H_2O_2 in vivo at about 10^{-11} and 10^{-9} M respectively (Forman and Kennedy, 1974, Forman and Wilson, 1982). Mitochondrial ROS production is intimately linked to membrane potential. Indeed, at high membrane potentials, protons cannot be pumped out of the matrix (against the electrochemical proton gradient) by the electron transport chain. Therefore, the electron transport slows, thus

resulting in the production of intermediates able to reduce O_2. These processes increase ROS production that may be reduced, modestly, by the mitochondrial inner membrane through the uncoupling proteins (UCPs) (Skulachev, 1996, Kim-Han et al., 2001, Votyakova and Reynolds, 2001). Moreover, ROS can also react with nitric oxide (NO) to produce reactive nitrogen species (RNS) (Patel et al., 1999). There are several neurodegenerative disorders (NDDs) identified so far, that have been reported to be associated with stress and mitochondrial dysfunction, which include Alzheimer's disease, Parkinson's disease, Huntington's disease, and Amyotrophic lateral sclerosis (Seo et al., 2010; Uttara et al., 2009). Overproduction of ROS, as by products generated from electron flow through the respiratory chain, usually occurs during normal respiration where the 1 – 6 % of the oxygen reduced by mitochondria is converted to superoxide anion at the level of complex I or at the level of ubiquinone (Chance et al., 1979, Boss et al., 1997, Bechmann et al., 2002).

As regard UCPs, they appear to be important to several metabolic processes (Klingenberg and Echtay, 2001). For instance, UCP2 acts as a protonophore and is activated by superoxide anions from within the matrix of the mitochondria (Echtay et al., 2002). Several studies have reported roles for UCPs in modulating ROS production (Negre-Salvayre et al., 1997, Arsenijevic et al., 2000, Sullivan et al., 2003, Sullivan et al., 2004a, Sullivan et al., 2004b). For instance, leptin-deficient mice have decreased levels of UCP and increased ROS production in macrophages (Lee et al., 1999). Overexpression of UCP2 decreases cell death following H_2O_2 exposure and ROS production (Li et al., 2001). This protein is express in various part of the brain and may play a role in neuroendocrine, behavioral, autonomic functions and metabolic processes (Horvath et al., 1999; Diano et al., 2000; Richard et al., 2001)

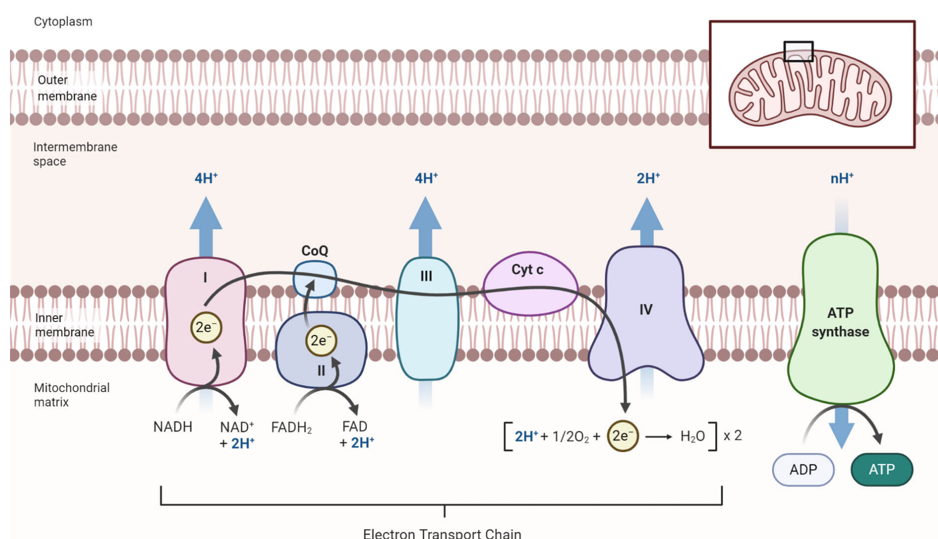

FIGURE 1: Mitochondrial electron-transport chain. (Figure modified from Microbe notes, Saptoka, 2021)

Diabetes mellitus induces oxidative stress and mitochondrial dysfunction

Hyperglycemia results in increased enzymatic conversion of glucose to the polyalcohol sorbitol, with concomitant decreases in NADPH and glutathione (Brownlee, 2001). The resulting loss of antioxidant reducing equivalents lead to enhanced sensitivity to oxidative stress associated with intracellular reactive oxygen species (ROS). The production of ROS is promoted by glucose through a combination of free radical generation and impaired free-radical scavenging. Hydrogen peroxide is produced by the action of superoxide dismutase on superoxide (O_2-), which is itself generated by increased oxidative metabolism of glucose in the mitochondria (Nishikawa et al., 2000b). Moreover, the sorbitol pathway compromises the glutathione cycle by consuming the proton donor NADPH. This reduces the capacity of glutathione peroxidase to metabolize hydrogen peroxide to water (Obrosova et al., 2002). Briefly, both systemic glucose and lipid metabolism converge in mitochondria generating most of the cellular energy (ATP) by coupling the tricarboxylic acid cycle (TCA) cycle with oxidative

phosphorylation (OXPHOS). Mitochondrial oxidative phosphorylation generates the energy necessary to fuel cellular function whereas the disfunction of mitochondria lead to reduced ATP production, impaired calcium buffering and formation of ROS (Beal et al., 2005). Acetyl-CoA generated from glycolysis (glucose) and fatty acid β-oxidation (lipid) enters the TCA cycle in the mitochondrial matrix, in which the substrates are oxidized with the formation of CO_2, NADH and $FADH_2$. The electron from NADH and $FADH_2$ are taken up by the respiratory chain complexes which generates an electrochemical gradient and drives the electron to ATP generation in the complex V. Mitochondrial metabolism is responsible for the major energy supply to vital cell functions including the maintenance of transmembrane ion gradients, protein synthesis, and vesicular transport (Wallace, 1999). Normally, only 0.1% of total oxygen consumption leaks from the respiratory chain to generate ROS. However, during the diabetic disease the hyperglycemia increase the electron flux through the mitochondrial electron transport chain. Consequently, there is an increase of the ATP/ADP ratio and hyperpolarization of the mitochondrial membrane potential. This high electrochemical potential difference generated by the proton gradient leads to partial inhibition of the electron transport in complex III, resulting in an accumulation of electrons to coenzyme Q. In turn, this drives partial reduction of O_2 to generate the free radical anion superoxide (Nishikawa et al., 2000a; Brownlee, 2001).

Hyperglycemic status reduces antioxidant levels and concomitantly increases the production of free radicals. These effects contribute to tissue damage in DM, leading to alterations in the redox potential of the cell with subsequent activation of redox-sensitive genes (Muriach et al., 2014). As a result of its high oxygen consumption rate, abundant lipid content, and the relative paucity of antioxidant enzymes as compared to other tissues, the brain is especially vulnerable to oxidative stress damage. Nevertheless, under normal condition, a balance exists between the production of ROS and the antioxidant mechanisms (Bala et al., 2006), when this balancing mechanism fails, such as in diabetes, oxidative insults

and therefore ROS may contribute to neurodegenerative processes (Jackson et al., 1994, Dugan et al., 1995, Yuan, and Yankner, 2000). In agreement, *Cardoso and collaborators* (2013), have shown that hippocampal mitochondria of STZ rats presented higher levels of lipid peroxidation, a marker of oxidative stress. Together with this data, the activity of some proteins involved in the antioxidant defense was altered by diabetic disease. Indeed, the activity of superoxide dismutase 2 (SOD-2) (i.e., the mitochondrial isoform of an antioxidant enzyme) was decreased after three months of diabetes (i.e., long-term). However, the activity of glutathione disulfide, which balances the production of H_2O_2, is increased while the glutathione-to-glutathione disulfide ratio (i.e., an index of oxidative stress) is decreased by long-term diabetes. This dichotomy between antioxidant molecules could be an attempt to overcome the decreased SOD-2 activity to maintain unchanged the H_2O_2 levels (Cardoso et al., 2013).

Different studies have observed in STZ- model, that diabetes also increased the level of MDA, a significant marker for lipid peroxidation while the level of GSH, a potent endogenous antioxidant is reduced in the brain of diabetic rats (Minaz et al., 2018; Masola et al., 2018). These results suggest that the depletion of antioxidant enzymes with an increase in free radical generation is one of a key mechanism involved in diabetes-induced cognitive impairment.

Moreover, in the same experimental model of DM, it has observed that diabetes increases lipid peroxidation and mitochondrial superoxide anions associated with an altered mitochondrial respiratory function in the brain cortex of STZ rats (Ortix et al., 2013). Most recently, it has also observed that after one months of diabetes, in STZ model of DM, hyperglycemia induces a significantly reduction of complex I activity and an increase in the activity of the antioxidant enzyme thioredoxin reductase, which are related to decreased hydrogen peroxide generation, oxygen consumption and mitochondria-coupled hexokinase (mt-HK) coupled-to-OXPHOS activity via mitochondrial complex I (Silva-Rodrigues et al., 2020). They also demonstrated, for the first time, that T1DM increases respiratory parameters and mt-HK activity via mitochondrial complex II and provide evidence that early

progression of hyperglycemia, in brain tissue, changes the coupling of glucose phosphorylation at the level of mitochondria by rearranging the oxidative machinery of brain mitochondria towards complex II dependent electron harvest (Silva-Rodrigues et al., 2020).

ii) Neuroinflammation

Chronic hyperglycemia triggers an enhanced neuroinflammation that manifests as microglial activation and the increased release of important inflammatory factor, such as interleukins (ILs) and tumor necrosis factor (TNF), can directly result in synaptic impairment.

Neuroinflammation is defined as an inflammatory response within the brain or spinal cord. This inflammation is mediated by the production of cytokines, chemokines, ROS, and other secondary messengers. These mediators are produced by microglia and astrocytes in the CNS but also by endothelial cells, and peripherally derived immune cells (DiSabato et al., 2016). Microglia has a pivotal role in neuroinflammation. This is because the innate immune cells perform the primary immune surveillance and macrophage -like activities of the CNS such as the production of cytokines and chemokines. Indeed, most of the innate immune capacity of the CNS is mediated by microglia (*figure 2*).

These cells are resident CNS cells that live in both gray matter and white matter of the brain and spinal cord. Then, microglia represent 10% of the CNS population and they have an active role in immune surveillance. Among the immune-related activities mediated by microglia, there is the propagation of inflammatory signals that are initiated in the periphery (Dantzer et al., 2008). These responses are pivotal in the communication between the immune system and the brain. For example, during a phenomenon of disease or infection, microglia become “activated” and function like inflammatory cellular mediators. Depending on context, the production of chemokines and cytokines can facilitate the recruitment of leukocytes to the brain (Zhou et al. 2006). In general, microglial activation and the resulting increased expression of cytokines have the task of

protecting the CNS. However, amplified, exaggerated, or chronic microglial activation can lead to strong pathological changes and neurobehavioral complications such as depression and cognitive deficits (Norden and Godbout, 2013).

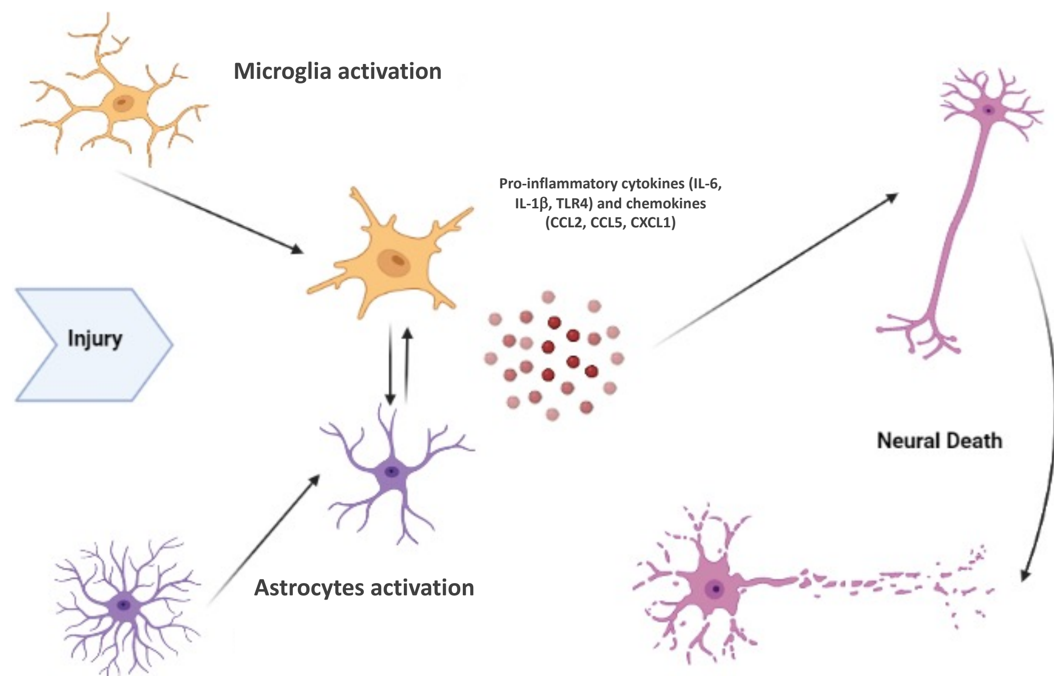

FIGURE 2: Neuroinflammation process in the central nervous system (figure created by biorender.com)

Neuroinflammatory signaling

Neuroinflammatory responses are mediated by several pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, chemokines (CCL2, CCL5, CXCL1), secondary messengers (NO and prostaglandins), and reactive oxygen species (ROS). Many of these mediators are produced by activated microglia and astrocytes, the resident CNS cells (Norden et al., 2016). Active microglia provide support, synaptic pruning, and immunological activities within the CNS (Schafer and Stevens 2013). Furthermore, enhanced neuroinflammatory signaling between T-cells and resident CNS cells are implicated in normal memory and learning (Ziv et al. 2006). These are examples that represent a degree of neuroinflammatory mediators which acts on other cells to influence development, physiology, and

cellular biochemistry. Pathological or highly destructive neuroinflammation is associated with activation of glia with significant release of cytokine and chemokine, infiltration of peripheral immune cells, increased blood-brain barrier permeability and breakdown (Hawkins and Davis 2005; Michael et al. 2015). There is also a primary event caused by the mechanical and physical damage of injury, infection, or insult. In addition, there can be ischemia, cell death, vascular occlusion, and other secondary inflammatory components from these insults. This type of neuroinflammation has been normally associated with stroke (Liesz et al. 2011), CNS infection (Goldman et al. 2001), or disease (Linker et al. 2002; Walter et al. 2007).

A notable chronic type of neuroinflammation is associated with Alzheimer's disease (Walter et al. 2007; Sokolova et al. 2009). Alzheimer's disease progression consists of protein misfolding, infiltration of peripheral immune cells, activation of CNS glia, neuronal damage and death, and then neuronal atrophy over time (Bucciantini et al., 2002; Sokolova et al., 2009). This degree of neuroinflammation is chronic, progressive, and increasingly destructive over time.

There is also a transient neuroinflammation which involves activation of resident glia and the release of important neuroinflammatory cytokines, such as IL-1β, IL-6 and TNFα. The induction of these neuroinflammatory markers can be induced as a part of CNS interpretation of peripheral infection of injury. In this situation, the activation of glia and consequently production of chemokines and cytokines lead to physiological and behavioral responses that are beneficial to the host organism (Imeri and Opp, 2009). It is important to underlie that this is a transient response and is not associated to classical consequences of chronic neuroinflammation, such as blood–brain barrier breakdown, significative infiltration of adaptive immune cells into the brain or cell death.

Neuroinflammation and diabetes

As explain before, chronic hyperglycemia induced neuroinflammation causing microglial activation and increased release of interleukins (ILs) and TNF, can directly result in synaptic impairments. Physiologically, microglia secrete factors that are able to regulate synaptic function in the juvenile as well as mature brain, after the establishment of synaptic connections. Among interleukins, IL-1β is primarily expressed by microglia and has been implicated in regulating long-term potentiation (LTP) in hippocampus (Williamson et al., 2013; Zhang et al., 2014). Aberrant IL-1β signalling furthermore underlies deficits in hippocampal LTP in animals lacking CX3CR1 (Rogers et al., 2011) but also in different experimental model of diabetes (Chabot et al., 1997; Zhang et al., 2020). Microglia may also be contributing to homeostatic plasticity. TNF-α released by glia, is required for synaptic scaling following a long-term reduction in activity (Stellwagen et al., 2006). Given that TNF-α expression is highly enriched in microglia compared with other cells in the brain, microglia may mediate this effect (Zhang et al., 2014). In STZ-induced mice model, it has been observed that diabetic condition induced cognitive impairment link to a neuroinflammation processes, increasing the expression of TNF- α both in frontal cortex and in hippocampus of diabetic mice (Pei et al., 2017). According to this work, *Baluchnejadmojarad and collaborators*, observed in the same model of DM, that cognitive deficit, observed in STZ- group by Y-maze task, are in partly due to the increase of inflammatory cytokines such as TNF-α and TLR4 because the treatment with an anti-inflammatory molecule, S-allyl cysteine, ameliorate memory impairment *(*Baluchnejadmojarad *et al., 2017).* In addition, it has been shown that microglia activation mediated TLR4 also play an important role in neuroinflammation associated with cognitive dysfunction in high glucose conditions, including DM (Zhang et al., 2015).

iii) Aberrant synaptogenesis

In addition to oxidative stress and mitochondrial dysfunction as well as neuroinflammation, the development of cognitive deficits seems to be due also to aberrant synaptogenesis characterized by synaptic protein loss and synaptic structure impairments (Biessels et al., 1996; Xu et al., 2021; Kuhad et al., 2009; Gaspar et al., 2016).

Synaptogenesis is a process involving the formation of a neurotransmitter release site in the presynaptic neuron and a receptive field at the postsynaptic partners, and the precise alignment of pre- and post-synaptic specializations (Jin, 2005). Neurons differ from other cells in their ability to communicate with each other very precisely and over great distances using mechanisms of axonal conductivity and synaptic transmission. The region at which communication between a neuron and other cells takes place is the synapse. Synapses are morphologically distinct subcellular junctional structures, composed of a presynaptic terminal, a postsynaptic target and the synaptic cleft aligning pre- and post-synaptic specializations (Cowan et al., 2001; Pappas and Purpura, 1972). The presynaptic terminal is characterized by a cluster of synaptic vesicles surrounding the electron-dense membrane specializations; and the postsynaptic site contains densely packed ion channels and signal transduction molecules (De Camilli et al., 2001; Sorra and Harris, 2000; Zhai and Bellen, 2004). The molecules acting as messengers at the synapses are neurotransmitters. They are stored in the presynaptic nerve terminal in small organelles 40-50 nanometers in diameter called synaptic vesicles. These vesicles release their transmitters upon fusion with a specific region called “active zone”. Then, neurotransmitters bind to their receptors in the postsynaptic cell and the signal is propagated (*figure 3)*. The “main players” in the exocytosis processes via synaptic vesicles are the *synaptic vesicles proteins*. Among these proteins, the synapsins (i.e Synapsin I and II) represent 0.3 to 0.6% of total brain protein and 6% of the total protein of purified synaptic vesicles from cerebral cortex.

Synapsins are substrate of multiple kinases suggesting that they represent a point of convergence of various signal transduction pathways involving Ca2+, cAMP, and growth factors. Multiple site phosphorylation seems to be a mechanism for tight regulation of synapsin function. Different studies have highlighted that synapsin I plays a role in regulating neurotransmitter release, in particular by controlling the initial steps of exocytosis (Bahler et al., 1989; Benfenati et al., 1989; Schiebler et al., 1986). Two different pools of synaptic vesicles have been identified in nerve terminals, a pool at the "active zone" and a second pool of synaptic vesicles that can be mobilized for later release events (Valtorta et al., 1990). A model for the short-term regulation of neurotransmitter release has been proposed (Bahler et al., 1989) in which synapsin I acts as a link between synaptic vesicles and actin filaments. In the resting state, dephosphorylated synapsin I immobilizes the second pool of synaptic vesicles. Nerve impulses increase the intracellular Ca2+-concentration in the synapse and then active protein kinase 11. The kinase phosphorylates synapsin I on two sites in domain D, leading to a decrease in the number of synapsin I molecules crosslinking synaptic vesicles to cytoskeletal elements. Vesicles are released from the cytoskeleton and become available for exocytosis. As regard synaptophysin, it is the most abundant integral membrane protein of small synaptic vesicles (Wiedenmann et al., 1985). It constitutes 6 to 8% of the synaptic vesicle membrane proteins. Synaptophysin is one of the best substrates for tyrosine kinase(s) in the brain thanks to a long cytoplasmic COOH terminus with ten copies of a tyrosine-rich repeat which contain sites for tyrosine phosphorylation (Pang et al., 1988). Different from synapsins, the expression of synaptophysin is not confined to the CNS and PNS but also in neuroendocrine cells like the β-cells of the pancreas (Redecker et al., 1991; Reetz et al., 1991) and in the bronchial tracts (Navone et al., 1986; Wiedenmann et al., 1986). The role of this synaptic protein is not completely known but thanks to its four transmembrane regions located in cytoplasm, synaptophysin constituted with connexin a channel voltage sensitive. It has been also reported that the cytoplasmic tail of synaptophysin contains a Ca^{2+}-binding site (Rehm et al., 1986) and that it may

therefore be part of the Ca^{2+}-trigger mechanism that initiates opening of a fusion pore. Nevertheless, synaptophysin might be part of a fusion pore in synaptic vesicles either as a homo-oligomer or together with other membrane proteins on synaptic vesicles. Another important group of protein which regulated synaptic vesicle exocytosis is called SNAREs (soluble N-ethyl-maleimide sensitive factor attachment protein receptors). SNARE proteins are the core component of the exocytosis machinery and reside either in the presynaptic vesicles (v-SNARE) or the targeted plasma membrane SNARE proteins (t-SNARE). The v-SNARE protein consists of VAMP2 (vesicle-associated membrane protein 2, also called synaptobrevin 2) (Brunger et al., 2015; Jackson et al., 2008), whereas the t-SNARE proteins include syntaxin-1A and SNAP-25 (synaptosomal-associated protein, 25 kDa) (Sutton et al., 1998). Syntaxin is a membrane-anchored protein that plays a central role in membrane fusion. It contains a cytosolic domain and a type II single-transmembrane C-terminal domain. The N-terminal Habc domain of syntaxin is believed to interact with Munc18, while the SNARE domain can form a four-helix-bundle with SNAP-25 and VAMP2 for membrane fusion. Syntaxin could oligomerize via the transmembrane domain (Milovanovic et al., 2016) or the cytoplasmic domain (Sieber et al., 2006). The balance between the assembly and disassembly of syntaxin clusters could play a crucial role during synaptic transmission (Padmanabhan et al., 2020).

Different studies suggest that a problem in synaptic vesicle exocytosis led to neurodegenerative diseases (Logan et al., 2017).

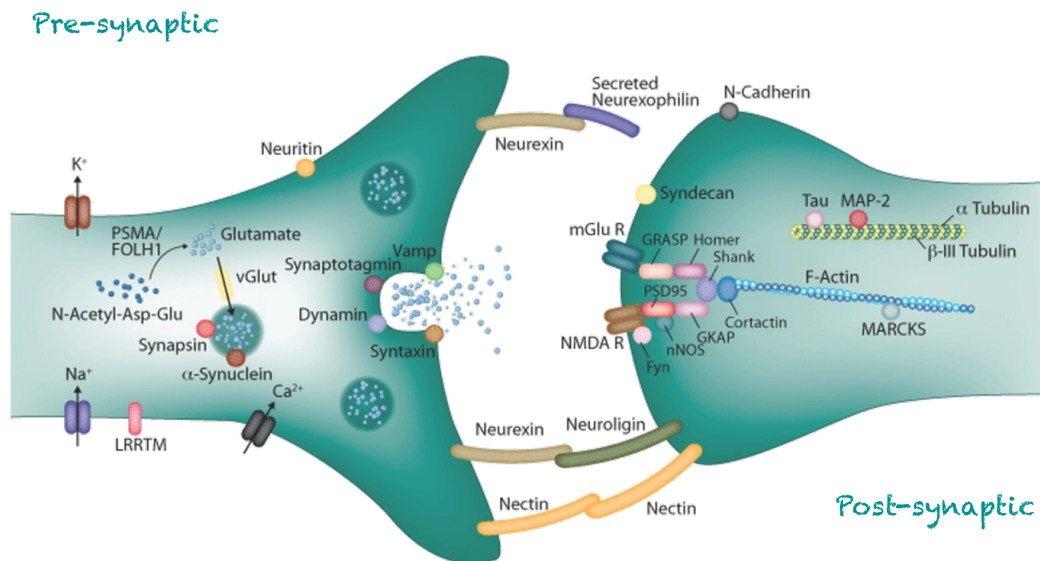

FIGURE 3: Representation of synaptic plasticity mechanisms.

Synaptic plasticity in learning and memory processes

Learning and memory are represented by greatly interconnected neural circuits; the connections are mediated by synapses that permit the neuron to pass an electrical or chemical signal to another neuron. The efficacy of a synapse is strengthened or weakened over time, and this phenomenon is called *synaptic plasticity* (Goto, 2022). Synaptic plasticity seems to be an important cellular substrate for memory and learning processes. This phenomenon has been linked with different learning-associated brain areas including the hippocampus, cerebral cortex, amygdala, and striatum (Frankland and Bontempi, 2005; Lüscher and Malenka, 2012; Tonegawa et al., 2018). Different areas of the brain exhibit various forms of synaptic plasticity. One of the important types of synaptic plasticity in the hippocampus, and then in systems memory consolidation, is the long-term potentiation (LTP) of excitatory synaptic transmission, a long-lasting experience-dependent strengthening in the efficacy of synaptic transmission (Lüscher and Malenka, 2012). Particularly, NMDA-type glutamate receptor (NMDAR)-dependent LTP in the hippocampal CA1 region and other forebrain regions has been well-characterized. Excitatory synapse is formed on a small protrusion on dendrites called dendritic spines. During LTP induction, there is an influx of Ca2+

into the postsynaptic compartment through NMDARs that results in the activation of calcium/calmodulin-dependent kinase II (CaMKII) producing subsequent phosphorylation of several proteins, including AMPA-type glutamate receptors (AMPARs) (Derkach et al., 1999). The phosphorylation of AMPAR subunits can cause an increase in the conductance of AMPAR channels. In addition, the increase in CaMKII activity contributes to the insertion of AMPARs, leading to potentiation of synapses (Hayashi et al., 2000). At the same time, new dendritic spines are formed, and the volume of existing ones increases (Engert and Bonhoeffer, 1999; Okamoto et al., 2004; Maletic-Savatic et al., 1999; Matsuzaki et al., 2004). NMDAR-associated Ca2+ influx also promotes synthesis of both mRNA and protein (Kandel et al., 2014). Changes in gene expression and protein synthesis are thought to contribute to postsynaptic structural changes, as well as to increased sensitivity to neurotransmitters leading to the long-term stabilization of synaptic transmission. For example, an increase in the postsynaptic scaffolding proteins PSD-95 and Homer1c has been shown to correlate with stabilization of synaptic enlargement (Meyer et al., 2014). The process underlying LTP described above completes within hours and involves the stabilization of changes in synaptic connectivity in the local circuits. This process is called synaptic consolidation which can be explained by protein synthesis-dependent transport of PSD scaffolding proteins to the synapses (Bosch et al., 2014; Frankland and Bontempi, 2005).

Diabetes mellitus alters synaptogenesis

Data obtained from different experimental model of DM, support the hypothesis that diabetic encephalopathy is characterized by aberrant synaptogenesis and neurochemical abnormalities leading to cognitive decline (Kuhad et al., 2007)) even though the exact molecular mechanism involved is still not well delineated. *Xu and collaborators* have observed, in mice STZ model, that synaptic proteins, synaptophysin and PSD-95 are decreased by diabetes in hippocampal neurons but the inhibition of mTOR and nuclear factor-kappa (NF-k) B increased their expression. The DM also induced damage of synaptic ultrastructure in the hippocampal CA1 region, including widened synaptic cleft and decreased

postsynaptic density (Xu et al., 2021). However, hyperglycemia significantly reduced the level of brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, which regulates the neuronal plasticity and survival associated with neuroinflammation (Nitta et al., 2002). Interestingly, in these studies (Nitta et al., 2002; Xu et al., 2021) the decrease of BDNF may also induce aberrant synaptogenesis and structural impairments of synapses.

Altogether, the observations collected from literature and reported in this elaborate, significantly supported the theory that the cognitive deficit which characterizes DE, is due to the concomitant presence of oxidative stress, altered function of the mitochondria, neuroinflammation and aberrant synaptogenesis in brain of diabetic animals. However, another factor possibly involved in the development of DE may be the sex steroid environment. Sex steroids are synthetized in the peripheral glands (i.e, sex steroid hormones) as well as directly in the nervous system (i.e., neuroactive steroids) and are important physiological regulators of the nervous function (Melcangi et al., 2008, Melcangi et al., 2016; Diviccaro et al., 2021; Giatti et al., 2012). Indeed, it is largely known that these steroid molecules are involved in brain function (Arevalo et al., 2015b; Baudry et al., 2013; Foy et al., 2008; Frankfurtand Luine,2015; Garcia-Segura et al., 1994; Kramar et al., 2013; McEwen and Woolley, 1994; Murphy and Segal, 2000; Ooishi et al.,2012) and cognition (Arevalo et al.,2015b; Celec et al., 2015; Colciago et al., 2015; Frankfurt and Luine,2015; Kramar et al., 2013; Velazquez-Zamora et al., 2012). On the other hand, neuropathological conditions included DM, affects their levels and consequently different steroid molecules have been largely demonstrated to exert neuroprotective effects (Melcangi et al., 2016; Giatti et al., 2019; Melcangi et al., 2014).

iv) Neuroactive steroids: steroid hormones and neurosteroids

Steroid hormones are lipid molecules synthesized in peripheral glands such as gonads or adrenal cortex and they interact with nuclear receptors as well as with membrane receptors (Brinton et al, 2008; Melcangi et al, 2008).
These molecules exert their effects through the interaction with nuclear receptors (Brinton et al., 2008, Melcangi et al., 2008). These receptors, named "*classical steroid receptors*", are localized in the cytoplasm and, when activated by binding to the hormone, translocate into the nucleus where they exert a regulatory action on the genome (Yamamoto, 1985). The activation of these receptors may explain the medium- and long-term effects of steroid hormones, like for example, the regulation of the secretion of hypophyseal hormones, or the sexual differentiation of brain circuits. However, different studies demonstrated that steroids may also induce short-term effects, suggesting the existence of other receptors, called "*non-classical steroid receptors"*, located within the membrane.
The steroid structure is characterized by a tetracyclic system of carbon atoms, called *cyclopentanoperidrofenantrene* or *sterane*. It consists of four rings, which of them one pentameric- and three esameric-carbon cycle, the typical basic structure of cholesterol. Neuroactive steroids may be divided into different groups: androgens (testosterone and derivatives), estrogens (estradiol), progestogens (progesterone and derivatives) and corticosteroids, which in turn include glucocorticoids (cortisol) and mineralocorticoids (aldosterone) (*figure 4*).
Several data from literature have demonstrated that steroid hormones exert their activity on the CNS and PNS, controlling important physiological processes such as memory, behavior, the brain sexual differentiation and reproduction (Fink et al, 1991; McEwen,1981, 1994) with mechanism which usually considered the classical endocrine mechanisms. However, steroid hormones released by peripheral steroidogenic tissues, are not the only endocrine molecules acting on the nervous system. Indeed, it was demonstrated that the nervous system is able to synthesize

steroid molecules (Corpechot et al, 1981; Giatti et al, 2015). Indeed, significant amount of dehydroepiandrosterone (DHEA), pregnenolone (PREG) and their sulfate esters (PREGS and DHEAS) in the mammalian brain was observed after 15 days of adrenalectomy and orchidectomy (Corpechot et al, 1983). *Baulieu and collaboratores*, called these molecules "neurosteroids". Thus, neurosteroids are steroids directly synthesized by neurons and glial cells, which have the ability to regulate the nervous system function, with an autocrine/paracrine mechanism of action (Baulieu, 1998). Since the nervous system is a target for two different pools of steroids, steroid hormones and neurosteroids, (i.e., one coming from the peripheral glands and the second one originating directly in the nervous system, respectively) in many circumstances, it is difficult to assign which is the responsible of the effects on the nervous system. Therefore, it has been coined the term "neuroactive steroids" that includes steroid hormones and neurosteroids (Paul and Purdy).

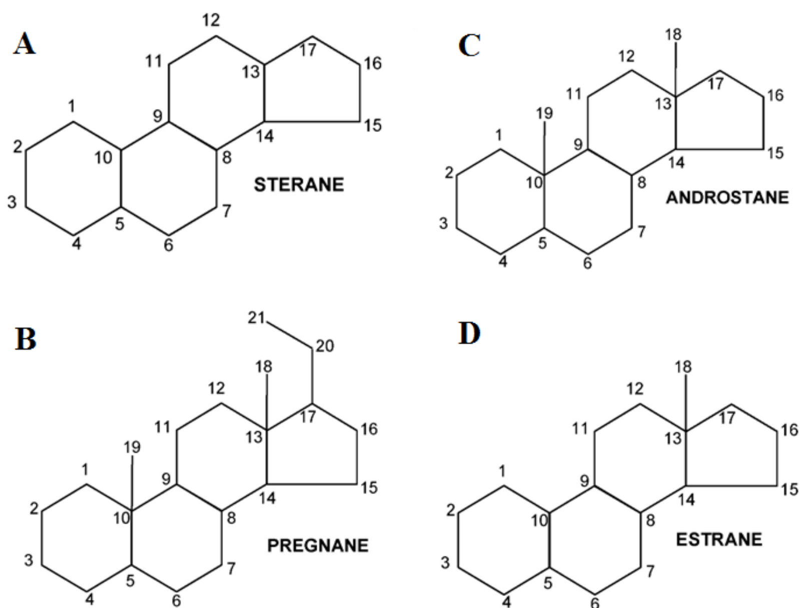

FIGURE 4: Chemical structure of steroids. **A**) sterane or cyclopentanoperidrofenantrene, basic structure of all steroidal compounds; **B**) pregnane, the core characterizing all C21 steroids such as progestogens and corticosteroids; **C**) androstane, characteristic nucleus of C19 steroids such as androgens; **D**) 42-estrane, characteristic core of C18 steroids such as estrogens. (Figure modified by (Mensah-Nyagan et al, 2009).

Biosynthesis and metabolism of steroids in the nervous system

The demonstration of the biosynthesis of these steroids in the nervous system comes from evidence that underline the expression and biological activity of the key enzymes of steroidogenesis in nervous cells. These enzymes are involved in the translocation of cholesterol from intracellular stores to the inner mitochondrial membrane, which is the first step of synthesis. This carriage occurs through a molecular complex constituted by the steroidogenic acute regulatory protein (StAR), the translocator protein 18 kDa (TSPO) and the voltage-dependent anion channel protein (VDAC) (Lavaque et al, 2006b; Papadopoulos et al, 2006a; Papadopoulos and Miller, 2012; Sierra et al, 2003). Furthermore, there are numerous key enzymes involved in the steroids synthesis as well as in the conversion of these into neuroactive metabolites. Initially, in the inner mitochondrial membrane, cholesterol is actively converted into pregnenolone (PREG) by the cytochrome P450 side-chain cleavage (P450scc) enzyme (Compagnone et al, 1995; Le Goascogne et al, 1987) (See figure 5). This is the first, rate-limiting, and hormonally regulated step in the synthesis of these molecules. Thus, it is the expression of CYP11A1 (i.e., P450scc gene) and the respective presence of the protein that makes a cell "steroidogenic" and able to produce steroids de novo, as opposed to modifying steroids produced elsewhere, which occurs in many types of cells. Then, PREG diffuses into the cytosol where is further transformed into progesterone (PROG) or dehydroepiandrosterone (DHEA) in the endoplasmic reticulum (Figure 5). Indeed, further steroidogenic enzymes, such as cytochrome P450c17 (P450c17), 3β-hydroxysteroid dehydrogenase (3β-HSD), 5α-reductase (5α-R), 3α-hydroxysteroid oxidoreductase (3α-HSOR), 3β-hydroxysteroid oxidoreductase (3β-HSOR), 17β-hydroxysteroid dehydrogenase (17β-HSD) and aromatase (ARO) were also identified (Baulieu, 1999; Compagnone et al, 2000; Melcangi et al, 2008a; Mensah- Nyagan et al, 1999; Schumacher et al, 2003).

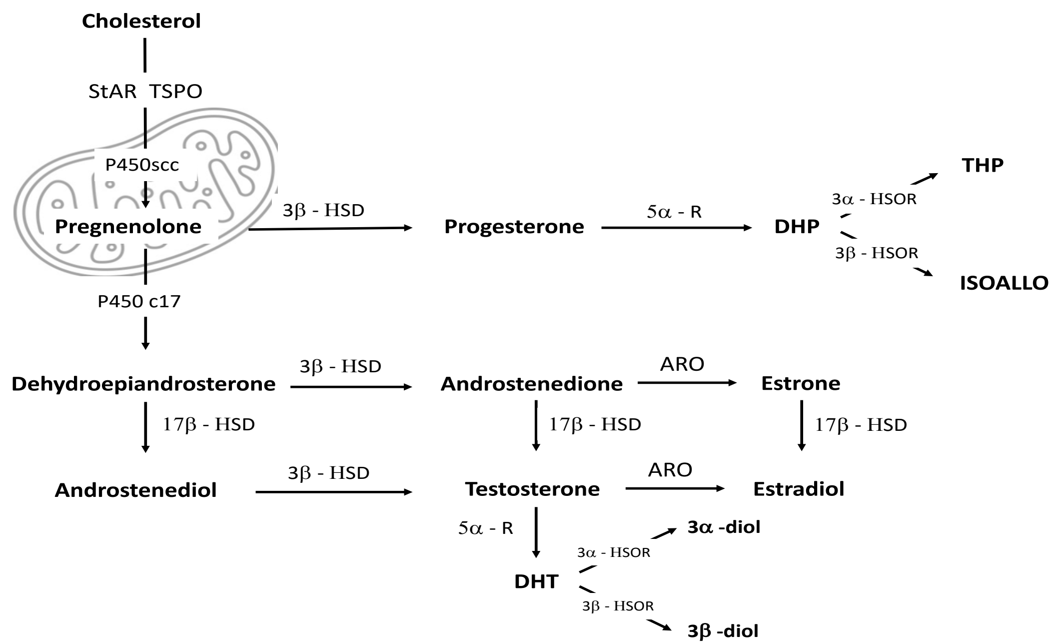

FIGURE 5: Schematic representation of steroidogenic pathway.

Mechanism of action of neuroactive steroids: classical and non-classical receptors

As previously described, neuroactive steroids are actively metabolized by several enzymes, such as 5α-R, 3α-HSOR and 3β-HSOR. These enzymatic steps exert an important role in the mechanism of action of neuroactive steroids, since active metabolites of these molecules exert their effects by a variety of mechanism, including the activation of *classical steroid receptors* signaling mediated by progesterone receptor (PR), androgen receptor (AR) and estrogen receptors (ERs), which are present in different isoforms, such as A and B or α and β (see for review (Melcangi et al, 2008a). On the other hand, some of these metabolites may also exert their effects by binding to membrane receptors, such as gamma aminobutyric acid (GABA)-A and GABA-B receptors, glutamate N-Methyl-D-aspartate (NMDA) receptor, AMPA and kainate subunits, membrane PROG receptors (mPRs) and pregnane X receptor (PXR) (Almey et al, 2014; Melcangi et al, 2008a; Nag and Mokha, 2014; Qin et al, 2015; Schumacher et al, 2014).

Notably, dihydroprogesterone (DHP) as progesterone (PROG), bind the classical steroid receptor, the PR (Melcangi et al, 2008a). Tetrahydroprogesterone (THP) is a potent ligand of a non-classical steroid receptor, such as the GABA-A receptor (Belelli and Lambert, 2005; Lambert et al, 2003), while isoallopregnanolone (ISOALLO) antagonizes the THP effect (Melcangi et al, 2008a). Similarly, DHT like its precursor T, interacts with AR, instead, 3α-diol is a GABA-A receptor agonist, while 3β-diol is an estrogen receptor (ER)β agonist (Handa et al, 2008; Melcangi et al, 2008a).

On the other hand, it is important to underline that also neuroactive steroid substrate, such as DHEA and PROG have been recently reported to exert effects via non-classical steroid mechanisms. For instance, in case of DHEA, it is unclear whether it is able or not to interact with AR (Lu et al, 2003; Mo et al, 2009; Mo et al, 2006) but some observations indicate a role for GABA-A receptors in DHEA signaling (Maninger et al, 2009). Moreover, in case of PROG, also in the nervous system, like in other tissues, classical PR may move to the cytoplasm or the plasma membrane and interacts with components of intracellular signaling pathways, such as kinases. This is also the case of ERs (Schumacher et al, 2014). Furthermore, picomolar concentrations of PREG sulfate are able to increase the intracellular response to glutamate at synaptic NMDA receptors via the phosphorylation of cAMP response element-binding protein (Smith et al, 2014). Thus, metabolic conversion of PROG and T into their metabolites may differently modulate the mechanism of action of their precursor molecules by recruiting specific pathways of central nervous system.

Both neurons and glial cells express the estrogenic receptors, represented by the two nuclear receptor isoforms, ERα and ERβ (Brinton et al, 2008; Melcangi et al, 2008a; Melcangi et al, 2001). However, 17β-estradiol also mediates rapid signaling events via pathways that involve transmembrane ERs, such as G-protein-coupled ER 1, (GPER, formerly known as GPR30) (Prossnitz et al., 2011). GPER mRNA and protein expression have been found both in the CNS and PNS of male and female rodents, including in the hypothalamus, hippocampus, and midbrain, as well as

the dorsal root ganglia and spinal cord (Brailoiu et al., 2007; Wang at al., 2008; Hewitt et al., 2005). Recent studied show the expression of GPR30 in microglia (Habib et al., 2014; Zhang et al 2013). This receptor mediates the acute neuroprotection of estrogen and regulates neuronal functions, such as hippocampal synaptic plasticity and neurotransmitter release (Briz et al., 2015).
Not all types of glial cells, however, express the same repertoire of steroid receptors and their expression varies depending on the state of activation of cells, localization, and stage of development. For example, the ERα receptor is expressed in microglial cells whereas ERβ, AR and PR do not appear to be expressed in physiological conditions (Sierra et al, 2008; Sierra et al, 2007). Similarly, changes in expression and metabolism of steroid receptors in response to different insults have been observed. For example, in rats, brain lesion induces the expression of ERα in vimentin and GFAP immunoreactive astrocytic cells, or the AR in microglia (García-Ovejero et al, 2002).

Diabetes mellitus and neuroactive steroids

Experimental diabetic neuropathy and experimental diabetic encephalopathy show similar features to human complications (Biessels et al., 1999, Bianchi et al., 2004, Biessels and Gispen, 2005, Mastrocola et al., 2005, Beauquis et al., 2006, Stranahan et al., 2008, Zhang et al., 2008, Alvarez et al., 2009). Different findings from experimental models of diabetic neuropathy and diabetic encephalopathy indicate that neuroactive steroids are protective agents (Aragno et al., 2002, Yorek et al., 2002, Saravia et al., 2004, Saravia et al., 2006, Veiga et al., 2006, Leonelli et al., 2007, Beauquis et al., 2008, Roglio et al., 2008, De Nicola et al., 2009). For instance, treatment with PROG, or its 5α-reduced metabolite, DHP, counteracts the increase in the number of fibers with myelin infoldings observed in the sciatic nerve of STZ- treated rat (Veiga et al., 2006). Moreover PROG, T and their derivatives (e.g. DHP, THP, DHT and 3α-diol) or DHEA influence a variety of biochemical and functional parameters, including nerve conduction velocity, thermal threshold, skin innervation density, Na^+,K^+-ATPase activity and expression

of myelin proteins, which are affected in STZ-treated rats (Yorek et al., 2002, Leonelli et al., 2007, Roglio et al., 2008). In the CNS, DHEA protects the hippocampus from diabetic damage reducing NF-kB nuclear translocation (Aragno et al., 2002).

On the other hand, diabetic condition affects levels of neuroactive steroids and also induces structural and neurophysiological changes in CNS (i.e., DE) (McCall, 2002). As mentioned previously, they are associated with cognitive deficits and increased risk of dementia, stroke, cerebrovascular disease, and psychological disorders which are pathologies showing sex-specific features (Biessels et al., 2002, 2008; Gispen and Biessels 2000; Jacoboson et al., 2002; Kodl et al., 2008). Different studies have reported, in a long-term model of diabetes (i.e., three month of diabetes), that alterations of neuroactive steroid levels in three brain areas of the CNS occurs. In particular, cerebral cortex (CTX), cerebellum (CB) and spinal cord (SC) were assessed. Data reported decreased levels of several neuroactive steroids such as PREG, PROG, DHP, THP, ISOALLO, T, dihydrotestosterone (DHT), 3α-diol in the CTX of STZ rats. Moreover, also the neuroactive steroids in the CB of diabetic rats were altered in particular, the levels of PREG, DHP, T and DHT were affected (Pesaresi et al., 2010b; Mitro et al., 2012) In addition, DE shows sex differences in the progression, incidence and severity depending on the pathology associated (Andersen et al., 1999; Farace and Alves, 2000; Fratiglioni et al., 1997; Kaye, 2008; Marcus et al., 2008) and altered levels of neuroactive steroids (Calabrese et al., 2014; Giatti et al., 2018; Pesaresi et al., 2010 a,b). Different studies (Pesaresi et al., 2010b; Giatti et al., 2018) have shown that, in the STZ-model, after three months of diabetes (long-term diabetes) a decrease in the levels of PREG, PROG, dihydroprogesterone (DHP), a PROG metabolite, T and 3alpha-diol (a T metabolite) occurred in plasma and in different areas of brain such as cerebellum and cerebral cortex. In the cerebral cortex, the levels of these neuroactive steroids are not impacted by diabetes in a sex dimorphic way. In contrast, in cerebellum, PROG levels are different between male and female rats (Pesaresi et al., 2010b). Most recently, *Romano and collaborators* (Romano et al.,

2017; Romano et al., 2018) have evaluated neuroactive steroid levels after one month of diabetes (short-term) in cerebral cortex and hippocampus of male STZ-rats. They reported that the levels of PREG and other neuroactive steroids (i.e., PROG, THP, T, DHT and 3α-diol) are decreased in brain areas considered, but not in plasma (Romano et al., 2017; Romano et al., 2018) suggesting that short term diabetes might affect directly the neurosteroidogenesis in male rats. They confirmed this hypothesis, observing a decreased expression of P450scc, as well as alteration in the homeostasis of neurosteroidogenic substrate (i.e., cholesterol) and the functionality of mitochondria (i.e., subcellular organelles where the PREG synthesis occurs) (Romano et al., 2017; Romano et al., 2018). Interestingly, one month of diabetes decreased the level of free cholesterol in the cerebral cortex of diabetic rats while in hippocampus a significantly increase of their levels was observed. These results suggest that short-term diabetes has specific effects depending on the brain region considered in male animals (Romano et al., 2017; Romano et al., 2018). However, no observation on female diabetic rats, after one month of DM, have been reported so far. It is extremely interesting explore the steroid environment linked to cognitive abilities in this sex, since diabetic encephalopathy shows some sex-dimorphic features.

A possible role of gut microbiota in the pathogenesis of T1DM

Diabetic encephalopathy observed in T1DM is associated with dysbiosis. The relationship between T1DM and gut microbiota was first investigated in the eighties in non-obese diabetic (NOD) mice (Suzuki 1987).

As mentioned before, T1DM has a multiple etiology due to the interaction of environmental and genetic factors that induce a deficit in the secretion of hypoglycemic hormone, insulin and/ or a reduction in its biological activity. A variety of factors, including viruses, chemical, commensal bacteria, and diet have been proposed to contribute to the increased risk of developing this metabolic disorder. In the last years, gut microbiota has been proposed as an important factor in T1DM pathogenesis (Gulden et al., 2015; McLean et al., 2015; Knip et al., 2016). The importance of intestinal microbiota in T1D etiology is further suggested by the differences in gut microbiota composition observed between individuals with autoimmune T1D and healthy controls that may influence the etiology and the progression of the disease. In particular, it has been shown that Firmicutes/Bacteroides ratio is reduced in T1DM patients, furthermore, Bacteroides and Clostridium cluster XIV a are more abundant in contrast to Lactobacilli, *Bifidobacteria*, and *Prevotella* which are less represented in gut microbiota of T1DM subjects (Murri et al., 2013; Giongo et al., 2011; de Goffau et al., 2013; Richardosn et al., 2014). Also in animal models, particularly in two rodent model, the NOD mouse, and the bio-breeding diabetes prone (BB-DP) rat, it has been reported interesting evidence between T1DM and gut dysbiosis. For example, an increased abundance of *Lactobacillus* and *Bifidobacterium* was detected in the BB-DR samples, while *Bacteroidetes, Eubacterium*, and *Ruminococcus* were more abundant in bio-breeding diabetes resistant (BB-DP) rats (Roesch et al., 2009). However, in NOD mouse, culture independent method revealed a relative increase in the abundance of a single species, *Akkermansia muciniphila*, while a depletion of many major genera *of Gram-positive* and *Gram-negative* microbes was observed (Hansen et al., 2012).

The composition of the gut microbiota can be also modulated by diet and environmental factors. This modulation can induce the proper maturation of the immune system or result in gut dysbiosis and altered immune response, as it happens in T1DM. Moreover, gut dysbiosis can lead to the increase in gut permeability, inducing a local and systemic proinflammatory response that stimulates β-cell autoimmunity in predisposed subjects *(figure 6)*.

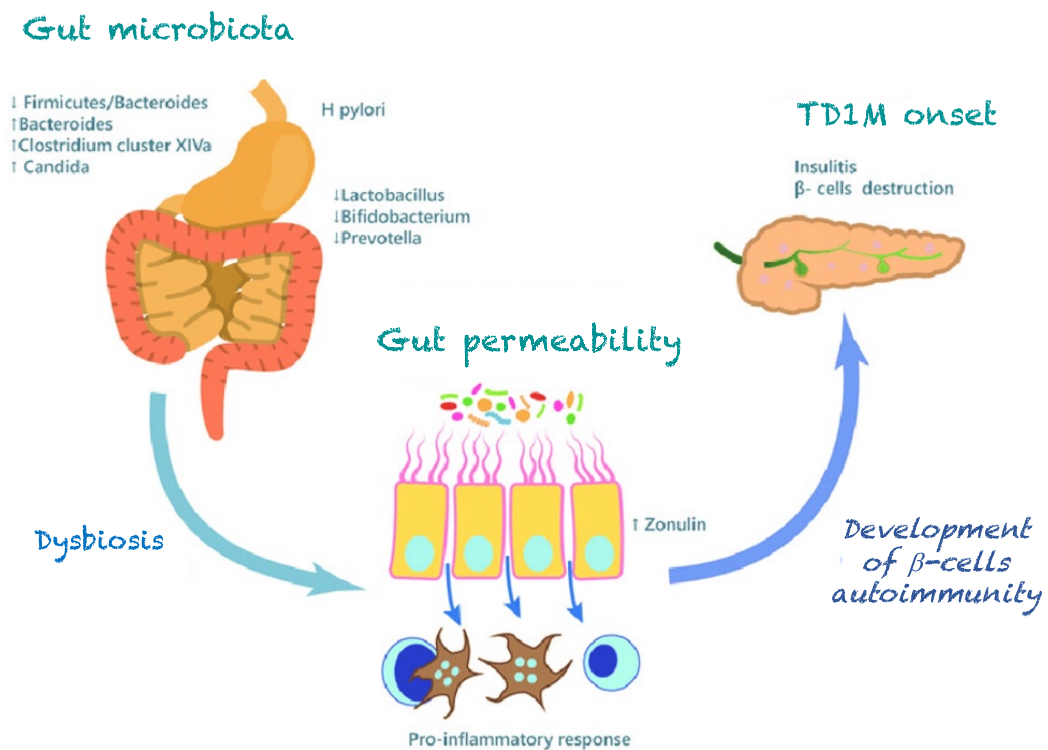

FIGURE 6: Role of microbiota in the pathogenesis of type 1 diabetes mellitus. (Figure modifies from Bibbò et al., 2017).

Intestinal Microbiota

The gastrointestinal tract is constantly colonized by a huge number of microorganisms, estimated at around 100 trillion, which are considered a virtual "organ" with endocrine function: the microbiota (Clarke et al., 2014; Li et al., 2021; Morris & Ridlon, 2017). The microbiota consists mainly of bacteria, but also viruses and fungi. These microorganisms have co-evolved with their host (Adak & Khan,

2019; Dinan & Cryan, 2017; Karakan et al., 2021; Li et al., 2021) and are numerically 10 times more than our cells and encode genes 150 times more than our genome (Clarke et al., 2014; John F. Cryan & Dinan, 2012, 2015; Dinan et al., 2015; Dinan & Cryan, 2017; Farmer et al., 2014; Moloney et al., 2014). The total weight of microorganisms in the adult human intestine weigh almost as much as the human brain (Clarke et al., 2014; Dinan et al., 2015; Dinan & Cryan, 2017; Li et al., 2021). Bacterial density and their metabolic abilities are higher at the level of the cecum and colon, and the composition varies along the entire gastrointestinal tract (GI) (Colldén et al., 2019; Farmer et al., 2014). *Firmicutes* (such as *Lactobacillus*, *Clostriudium*, *Enterococcus*) and *Bacteroidetes* (including *Bacteroides*) are the *phyla* predominant in our intestines (Kelly et al., 2016).

The relationship between the host and its microbiota can be defined as a 'mutual relationship': composition, health and functionality of the intestinal microbiota depend on the host and, in turn, to the functions of the microbiota as an endocrine organ have significant effects on host health. Among the functions performed by microbiota for maintaining of the guest's health, there are the digestion of particular foods such as complex non-digestible polysaccharides, the removal of pathogens and the synthesis of essential vitamins and nutrients for the individual (Adak & Khan, 2019; Bouguen et al., 2015; John F. Cryan & Dinan, 2012; Dinan et al., 2015; Farmer et al., 2014; Overby & Ferguson, 2021; Sittipo et al., 2018). The composition and the microbiota diversity are essential for maintaining homeostasis. In fact, the microbiota is a source of numerous metabolites that through systemic circulation can have significant effects on the physiology of the individual (Clarke et al., 2014; Colld. et al., 2019).

The intestinal microbiota has the ability to contribute to the exacerbation of several gastro-intestinal diseases, including irritable bowel syndrome (IBS; Osadchiy et al., 2019), inflammatory bowel disease and colon cancer (Yoon & Kim, 2021). In addition, variation in microbiota composition seems to be related with extra-intestinal diseases, among which obesity (Li et al., 2021; Osadchiy et al., 2019), diabetes, neurodegenerative diseases (Cani et al., 2021; Giatti et al., 2020).

The microbiota does not remain the same throughout our life. In the prenatal stage the fetus is practically sterile. Subsequently, the passage into the vaginal canal, another bacterial source, turns out to be the starting point for the formation of the microbiota in the newborn. There are several factors which modulate the newborn microbiota including use of probiotics and antibiotics, environmental factors, diet, maternal microbiota, stress, and mode of childbirth (Clarke et al., 2014; John F. Cryan &Dinan, 2012, 2015; Dinan et al., 2015; Farmer et al., 2014; Moloney et al., 2014; O'Mahony et al., 2015; Osadchiy et al., 2019). The child's microbiota is characterized by a low diversity (Dinan et al., 2015; Farmer et al., 2014) but with the time, the architecture of intestinal microbiota becomes more stable and more complex than the neonatal period.

Nevertheless, the intestinal microbiota of adults still exhibits plastic capacity, preferentially influenced by nutritional factors, which allow suddenly alterations in its composition (O'Mahony et al., 2015). But there are also other factors that can participate in the variation of the microbiota such as sex, age, nationality of the individual, infections, use of antibiotics, exposure to stress, probiotics, and prebiotics (John F. Cryan & Dinan, 2012). However, the invariability of bacteria belonging to three different enterotypes has recently been observed: *Prevotella*, *Ruminococcus* and *Bacteroides*. Indeed, they seem to be independent of factors such as gender, age, nationality, and body mass index (BMI; Clarke et al., 2014; John F. Cryan & Dinan, 2012; Dinan et al., 2015; Dinan & Cryan, 2017; Kelly et al., 2016; Moloney et al., 2014). Finally, when the individual became old, it has been observed another variation of microbiota composition (O'Mahony et al., 2015).

Gut brain-axis

The gut microbiota could interact not only locally with the gastrointestinal tract, but also with a whole series of networks involving organs and tissues far away from it. To allow this connection, several endocrine axes have been identified. Among these the most known is the microbiota-gut-brain axis, but communication is also due to the contribution of other axes, including for example gut-testis axis (Li et al., 2021) microbiota-immune-brain system (Audet, 2019).

Microbiota-gut-brain-axis

Communication between the microbiota and the central nervous system is possible through the microbiota-gut-brain axis. The observation of this communication allows to support the hypothesis that the intestinal microbiota plays a pivotal role in the modulation of brain functions and behavior. At the basis of this two-way communication there are neural, endocrine, immune, and metabolic pathways.

The general organization of microbiota–gut-brain axis involved the CNS, the hypothalamic–pituitary-adrenal axis (HPA) the neuroendocrine and neuroimmune system, sympathetic and parasympathic arms of the autonomic nervous system (ANS), the enteric nervous system (ENS) and the intestinal microbiota. These components interact to form a complex network with afferent fibers that integrate the structures of the SNC and fibers efferent to the intestinal smooth muscle (J. F. Cryan & O'Mahony, 2011; Dinan et al., 2015; Farmer et al., 2014; Moloney et al., 2014).

Communication between the intestinal microbiota and this axis occurs through different circuits, including neuropeptides secreted at intestinal level, vagus nerve, sensory nerves, and immune mediators (El Aidy et al., 2015). Through this two-way communication network, signals from the brain are able to affect the motor, sensory and secretory responses of the intestine and in turn, the viscera messages from the intestine can affect brain functions (J. F. Cryan & O'Mahony, 2011; Dinan et al., 2015; Farmer et al., 2014; Moloney et al., 2014). The vagus nerve (X cranial nerve) has both afferent and efferent divisions and plays a role fundamental in conducting signals from the brain to the intestine and vice versa. Many of the effects of the microbiota is able to bestow on brain functions which depend on the vagus nerve activation (Bravo et al., 2011; Dinan et al., 2015). In addition, there are mechanisms independent of the vagus nerve that play an important role in the interaction between microbiota and brain. For example, the immune system is another method of communication between intestinal microbes and the brain. Indeed, the microbiota and probiotic agents have direct effects on the immune

system. The innate and adaptive immunity collaborate in the maintenance of homeostasis at the level of the lumen between microbiotes and the host intestine. The production of inflammatory and anti-inflammatory cytokines in turn, can regulate the release of corticotropin releasing hormone (CRH), the main regulator of the HPA axis, which in turn is a two-way communication method (Dinan et al., 2015).

Moreover, microbiota is able to produce short-chain fatty acids (SCFA), bile acids and choline, essential for the health of the host and with important neuroactive properties. Bacteria also could generate numerous neurotransmitters and neuromodulators also present in the human brain: GABA, dopamine, acetylcholine, norepinephrine (Dinan et al., 2015). These neurotransmitters are able to act locally in the intestine, going to modulate the enteric nervous system (Karakan et al., 2021).

As mentioned before, T1DM is linked with alteration of gut microbiota (Giongo et al., 2011; Murri et al., 2013; Roesch et al., 2009). Moreover, risk of T1DM onset in childhood is higher in children delivered by Caesarean section (Cardwell et al., 2008), where there is also altered microbiota composition (Dominguez-Bello et al., 2010). Among factors that are involved in the development of diabetes, there are diet and gut microbiota (Rewers et al., 2016; Todd et al., 2007). Several studies have shown that the gut microbial composition differs between healthy hosts and hosts with T1DM or at risk of diabetes (Brugman et al., 2006; Roesch et al., 2009; Murri et al 2013; Giongo et al., 2011). interestingly, different experimental animal model of DM, such as Bio-Breeding (BB) rat and non-obese diabetic (NOD) mouse exhibit similar characteristics to human disease (Pearson et al., 2016). In Bio-Breeding diabetes-prone (BB-DP) rats, before the onset of T1DM, the composition of gut microbiota is markedly different between the rats that eventually will and will not develop T1DM (Brugman et al., 2006). Similarly*, Luiz et al*. observed a significant decrease in the number *of Lactobacillus, Bryantella, Bifidobacterium*, and *Turicibacter* in BB-DP rats, whereas the number of Bacteroides, Eubacterium, and Ruminococcus increased in BB-DP rats compared with the Bio-Breeding

diabetes-resistant (BB-DR) rats (Roesch et al., 2009). According with animal models, the gut microbial composition is also different between humans with T1DM and healthy humans. In a case-control study that included 16 children with T1DM and 16 healthy children, gut microbial composition showed marked differences between the healthy children and the children with diabetes. At the *phylum* level, the abundance of *Actinobacteria* and *Firmicutes,* and the ratio of *Firmicutes* to *Bacteroidetes* were all lower in the children with T1D than the healthy children (Murri et al., 2013). At the genus level, the healthy children had greater numbers of *Lactobacillus, Bifidobacterium, Blautia coccoides/Eubacterium rectale* group, and *Prevotella* in the gut, whereas children with T1D contained greater numbers of *Clostridium, Bacteroides*, and *Veillonella* (Murri et al., 2013). In addition to microbial abundance, the diversity and stability of intestinal microbiota are also associated with the development of T1DM (Simon et al., 2015; Endesfelder et al., 2014). Indeed, in experimental model of DM, STZ-induced T1DM, *Petterson and collaborators* have shown that, at T1DM onset, diabetic condition was associated with a shift in the *Bacteroidetes: Firmicutes* ratio while at genus level, increased proportions of lactic acid producing bacteria such as *Lactobacillus* and *Bifidobacterium* were associated with the later stages of diabetes. Moreover, microbial diversity was also reduced by diabetes. Particularly, α-diversity, a measure of microbiome diversity applicable to a single sample, is significantly decreased in diabetic rats (Patterson et al., 2015).

The intestinal surface barrier is one of the most important components of the innate immune system (Varaala et al., 2008), whereby DM compromises intestinal integrity (Carratù et al.,1999; Kuitunen et al.,2002; Sapone et al., 2006). Abnormalities of intestinal barrier, known as “leaky gut”, expose the immune system of gut to antigens (Vaarala et al., 2008). An impaired intestinal microbiota composition in children pre-diagnosed with autoimmune diabetes has previously been associated with altered microbial fermentation metabolite production (Brown et al., 2011). Butyrate producers such as *Eubacterium, Fusobacterium, Anaerostipes* and *Roseburia* were higher in the faeces of a control group of healthy

children. The producer of lactic acid such as Lactobacillus, Bifidobacterium and Streptococcus were higher in the faeces of children pre-diagnosed with autoimmune diabetes (Brown et al., 2011). Indeed, the fate of lactate is crucial to protect intestinal health thanks to its conversion in butyrate promoting synthesis of mucin (Barcelo et al., 2000; Burger-van Paassen et al., 2009; Finnie et al., 1995; Shimotoyodome et al., 2000) and tighter junctions (Peng et al., 2007, 2009). Mucin is a glycoprotein made by the host that maintains the integrity of intestinal epithelium (Brown et al., 2011). In addition, butyrate contributes to colonic health through its anti-inflammatory properties (Hamer et al., 2008; Louis & Flint, 2009; Pryde et al., 2002) and decreases bacterial transport across metabolically stressed epithelia (Louis & Flint, 2009), thus preventing the development of the so-called 'leaky gut'. Indeed, it has been shown, in experimental model of DM, that diabetic condition reduced butyrate-forming bacteria (i.e., *Butyricicoccus spp, Eubacteria rectal and Eubacteria intestinalis),* which are important fort the maturation of T-cell that produce anti-inflammatory markers (i.e., IL-10) (Bereswill et al., 2011; Park et al., 2015). This effect corresponds to reduced circulatory levels of IL-10 in diabetic mice in comparison with control groups (Noureldein et al., 2020). Furthermore, *Patterson and collaborators*, in their study observed that diabetes increased in the abundances of lactic acid producing bacteria such as *Bifidobacterium and Lactobacillus* in T1D rats, while butyrate was decreased in the caecum of T1D rats, compared with healthy controls supporting the hypothesis that DM has a negative effect on the integrity and functionality of gut (Patterson et al., 2015). It also known that the integrity of intestinal permeability is allowed by the proteins involved in the formation of the tight junctions between epithelial cells, which are able to regulate gut permeability, such as the claudine-1 (Cldn-1) and zonuline-1 (ZO-1) protein families (Panwar et al. 2021) and by the first barrier on the surface of the GI tract , the mucus layer, of which mucine-2 (Muc-2) is the most important component (Vancamelbeke et al. 2017, Yao et al. 2021). The role of intestinal permeability related with diabetic onset and progression is not fully clear but studied conducted in patients with T1DM (Pellegrini et al., 2017)

reported that the disruption of intestinal permeability is related with dysbiosis. Indeed, both humans and animal model studies highlight that disrupted gut microbiome has an important role in the pathogenesis of T1DM (Paun et al., 2016). As mentioned in the previous paragraph, diabetes mellitus also affected the nervous system (i.e., diabetic encephalopathy inducing neurophysiological and structural changes in grey and white matter of the brain (Hernandez-Fonseca *et al.* 2009, Zhou *et al.* 2013, Baptista *et al.* 2013, Pesaresi *et al.* 2010a, Kawashima *et al.* 2007). Indeed, diabetic encephalopathy is associated with cognitive deficit and increased risk of dementia, stroke, cerebrovascular and Alzheimer disease, as well as psychiatric disorders (Biessels & Reijmer 2014, Gispen & Biessels 2000). Interestingly, it has been proposed a role of gut-brain-axis in diabetic cognitive impairments (Xu et al., 2017). Indeed, it has been observed, in STZ-induced model, that T1DM induces cognitive disfunction in rats associated with alteration of the gut microbiota reducing the *Firmicutes*/*Bacteroidetes* ratio linked to a significantly weight loss of STZ group (Gao et al., 2019). Furthermore, *Zheng and collaborators* have been demonstrated that depletion of acetate-producing bacteria from gut microbiota accelerated cognitive impairment in diabetic mice (Zheng et al. 2021b, Zheng et al. 2021a).

Sex steroids influence the intestinal microbiota

To date, numerous evidence is emerging of how sex hormones can affect the intestinal microbiota and sexual dimorphism in the bacterial population. Numerous studies have been done to confirm the gender difference in microbiota composition, mainly on animal models, but also in humans (Poeran, 2017). It was observed that women exhibit a greater diversity of intestinal microbiota than men (Li et al., 2021; Tetel et al., 2018; Yoon & Kim, 2021). Different studies in gonadectomized animals to better investigate the functions of sex hormones on the microbiota, have been shown a variation between abundance and *phyla* ratio between gonadectomized and control animals (Audet, 2019; Yoon & Kim, 2021) and thus a possible involvement of sex hormones in the regulation of the intestinal microbiota. Moreover, it has also been a decrease in the differences between the

intestinal microbiota of females compared to gonadectomized males thus supporting the theory that androgens play a key role in inducing sexual dysmorphism in the intestinal microbiota (He et al., 2021; Li et al., 2021; Poeran, 2017; Tetel et al., 2018; Valeri & Endres, 2021; Yoon & Kim, 2021).The influence that androgens exert on the microbiota is also able to explain why in the prepuberal stage no significant differences in the intestinal bacterial population between males and females are observed (Jaggar et al., 2020) and, on the contrary, there is a change in the microbiota in women during pregnancy, specifically during the third trimester, a period when estrogen levels peak (Adak & Khan, 2019; He et al., 2021). Then, composition of the gut microbiota is influenced by sex hormones, but in turn it plays a pivotal role in the metabolism of steroids, as many bacteria have the ability to synthesize steroids, including the *Clustridium scindens* has the ability to convert glucocorticoids into androgens (Rizzetto et al., 2018). As extensively demonstrated, diabetes mellitus affects not only sex steroid hormones with impairment of the reproductive axis (Kim & Halter 2014, Babichev *et al.* 1998), but also the levels and synthesis of steroid molecules in brain areas (Romano *et al.* 2018, Romano *et al.* 2017). On the contrary, no observations on the possible effects of T1DM on gut steroids have been obtained so far. In addition, bacteria are essential in the colon as they deal with the diglucuronidation of androgens, going to increase levels of free DHT in both males and females. The ability of sex hormones to change microbiota, going to modify the population of particular bacterial species, can cause changes in the behavior of the individual. Indeed, different clinical studies have highlighted that the gut microbiota of patients affected by neuropsychiatric and neurological disorders shown a significant reduction of abundance and diversity. Moreover, preclinic studies have shown that the microbiota is able to affect behavior and specific gastrointestinal abnormalities associated with autism and other neurodevelopment disorders. Bravo and collaborators *(*Bravo et al., 2011) observed that *Lactobacillus rhamnosus* is able to modulate the behavior and the biochemistry of the CNS in healthy mice through the vagus nerve (Burnet, 2012;

McLean et al., 2012). From these studies it can be inferred that the microbiota is crucial to prevent social behavior, as these are important in neurodevelopment disease such as schizophrenia and autism (Desbonnet et al., 2014; Dinan et al., 2015). Indeed, it has been observed that 70% of patients with these diseases also present gastrointestinal symptoms, caused by a dysregulation of the gut-brain axis (Dinan et al., 2015; Dinan & Cryan, 2017). In addition, the influence of gut microbiota on the CNS has been demonstrated by the fact that, through the transfer of microbiota, the behavioral aspects of donor mouse have been transferred to adult germ-free mice of different breed (John F. Cryan & Dinan, 2015; Farmer et al., 2014).

Colon, a steroidogenic organ

An organ to be defined ‘steroidogenic’, or that is able to produce steroids *de novo*, must have the enzyme that catalyzes the limiting state of the steroidogenesis, the cytochrome P450scc. The classical steroidogenic organs are the gonads and the adrenal glands. The ovary can produce estradiol and progesterone, while testis mainly synthesize testosterone. In addition, the adrenal glands are source of glucocorticoids, mineralocorticoids, and sex hormones (Zheng et al., 2009).

In recent years, the presence of steroidogenic enzymes in different extra-gonadic tissues has been ascertained and therefore their ability to carry out steroidogenesis, among them the brain. In recent years, the presence of steroidogenic enzymes in some extra-gonadic tissues has been ascertained and therefore their ability to carry out steroidogenesis, among them the brain (Caruso et al., 2010; Giatti et al., 2019), the placenta (Noyola-Martínez et al., 2019), the heart (Young et al., 2001; Yu et al., 2002), the skin (Nikolakis et al., 2016; Thiboutot et al., 2003) and the colon (Diviccaro et al., 2020).

Synthesis and functions of glucocorticoids and sex hormones in the intestinal mucosa

Mucosa, in vertebrates, acts as a barrier against the invasion of pathogenic microorganisms, but it also represents an essential exchange surface between the

host and the environment (Bouguen et al., 2015). The intestinal epithelium, with an area of about 300 m2 in adults, is the largest mucosal-coated barrier in humans (Peterson & Artis, 2014). The high surface area of this epithelium is made possible by the organization of the endothelium in villi and crypts, which amplify its surface (Adak & Khan, 2019; Bouguen et al., 2015; Peterson & Artis, 2014).

The adrenal glands are the main source of endogenous glucocorticoids (GC), but recently it was discovered that the synthesis of these steroids is also attributable to other tissues, including the intestinal epithelium (Atanasov et al., 2008; Kostadinova et al., 2014). Indeed, the expression of the enzymes required for the synthesis of GC, P450scc and 11β-hydroxylase has been observed at the intestinal level (Kostadinova et al., 2014; Morris & Ridlon, 2017). In particular, these enzymes are preferentially expressed at the level of epithelial cells in the intestinal crypts. As regard the role of GC, since they are released in response to psychological or immunological stress, these steroids have a powerful immunoregulatory and anti-inflammatory activity. Moreover, thanks to these activities, it has been hypothesized that the glucocorticoids produced in the intestinal crypts are involved in the maintenance of the intestinal immune homeostasis and in the regulation of intestinal inflammatory mechanisms (Atanasov et al., 2008; Bouguen et al., 2015; Cima et al., 2004; Cole et al., 2019).

Steroidogenesis involves not only the production of GC but also other steroids such as mineralocorticoids and sex hormones. In fact, parallel to the production of GC in the intestine, the ability of intestinal epithelial cells to metabolize sex hormones has been described (Colldén et al., 2019). To synthesize androgens and estrogens from glucocorticoids some enzymatic steps are needed involving 17α-hydroxylase/17,20-lyase (P450c17) and 17β-hydroxysteroid dehydrogenase (17β-HSD). An additional enzyme is needed for estrogens: the aromatase. The 17β-HSD acts at key points in the synthesis of sex hormones. It has several isoforms but those most expressed in the intestinal mucosa are the isoform 2 and the isoform 4. This enzyme is able to catalyze the oxidative transformation of estradiol and testosterone into estrone and androstenedione, respectively (Bouguen et al.,

2015). Like to GC synthesis, estrogen metabolism, particularly of estradiol, is also predominant in the intestinal crypts of the colon and may therefore play a role in the growth of the epithelial cells of this organ. The expression of the estrogen receptor ER-β has been observed in the intestinal epithelial cells and signaling of this receptor is involved in cellular homeostasis and in the maintenance of the epithelial barrier of the colon. Indeed, the absence of this receptor, in KO mice, has been associated with an increase of epithelial cells prolifaration, a decrease in apoptosis and in the expression of cell adhesion molecules (Bouguen et a., 2015). Recently, also the expression of steroidogenic molecules and key steroidogenesis enzymes in the adult male rat colon has been demonstrated. Indeed, significant levels of PREG, DHEA, PROG and T and their metabolites were detected (Diviccaro et al., 2020). In this study (Diviccaro et al., 2020), it was possible to observe high levels of protein expression of P450scc and StAR, going to support the hypothesis that the PREG levels measured in the colon are originated by local synthesis. In addition, protein expression of the enzyme 3β-HSD and PROG derivatives was detected, indicating that local synthesis of PROG is also possible in this tissue. Then, in gut, it is not only possible to produce glucocorticoids, but also sex hormones. However, to determine the relative contribution of both local and peripheral steroidogenesis to the levels of steroids present in the colon, it is necessary to subject animals to gonadectomy. Recently, we have observed steroidogenesis along the gut-brain axis is affected differently by sex and peripheral steroid hormones, depending on the tissue considered. Interestingly, also gonadectomy significantly altered the gut microbiome, increasing alpha diversity and defining beta diversity (i.e., community composition) with a greater effect than either sex or sample substrate (Diviccaro et al., 2022)

AIM

Diabetes mellitus (DM), a chronic metabolic disorder, may induce neurophysiological and structural changes in the central nervous system (i.e., diabetic encephalopathy). Diabetic encephalopathy (DE) is one of the severe microvascular complications of diabetes, characterized by impaired cognitive and memory functions, and electrophysiological, neurochemical, and structural abnormalities. These alterations are associated with acute alterations in mental status due to poor metabolic control, decline in cognitive processes, increase risk of dementia, cerebrovascular and Alzheimer disease, psychiatric disorders and eating disorders.

Cognitive and memory impairments, that normally characterized DE, are associated with hippocampal dysfunction, but the mechanisms behind these impairments are not fully known. Data collected in literature, have demonstrated that the development of cognitive deficit seems to be the result of the concomitant presence of different processes such as oxidative stress, mitochondrial dysfunction, neuroinflammation and aberrant synaptogenesis in different brain areas of male rat models of type 1 DM (T1DM).

Another potential factor involved in the development of DE are steroids molecules because it is largely known that they are important regulators of the nervous function. Indeed, our previous data have demonstrated that brain levels of important neuroactive steroids (i.e., steroids produced in nervous system) are decreased by three months of DM (long-term diabetes) but also by one month (short-term) in male streptozotocin (STZ)-treated rats (i.e., an experimental model of type 1 diabetes mellitus).

Furthermore, diabetic encephalopathy presents differences in term of incidence, progression, and severity in two sexes. However, even if diabetic encephalopathy shows these sex-dimorphic features, no observations in female rats have been so far reported on these aspects.

Then, considering all these important issues which have been explored mainly in male animal models, we have decided to explore the impact of diabetes on memory abilities and mechanisms mentioned before in female STZ-rats because

we hypothesized that the potential mechanisms that cause cognitive deficits in female could be in part different from what has been observed in male diabetic animals.

For these reasons, in the present study we have explored the impact of one month of diabetes on: [1] memory abilities; [2] oxidative stress; [3] mitochondrial functionality; [4] synaptogenesis and [5] levels of neuroactive steroids in hippocampus and cerebral cortex of female STZ-rats.

T1DM is characterized not only by diabetic encephalopathy but also by dysbiosis. Recent studies have suggested a crucial etiopathogenetic role of intestinal microbiota in T1DM patients showing dysbiosis. Indeed, most of the studies conducted in experimental models of DM have reported changes in the diversity and composition of gut microbiota. In addition, the barrier disruption and the inflammatory signature observed in the duodenal mucosa of T1DM patients are linked to the pathogenetic process related to the dysbiosis.

Overall, it is unsurprisingly that a role of gut-brain-axis in diabetic cognitive impairment has been proposed. Indeed, it has been observed that the depletion of acetate-producing bacteria from gut microbiota facilitates cognitive impairment in diabetic mice. Furthermore, even if it has been extensively demonstrated that diabetes alters neuroactive steroids levels in brain areas, on the other hand, no observations on the possible effects of T1DM on gut steroids have been obtained so far. However, the interactions of the players of gut-brain axis, such as gut steroids, markers of gut permeability and microbiota, have been poorly explored in this pathology and, particularly in females.

Therefore, we have hypothesized that diabetes after one month alters gut steroidS levels and that these molecules could play a role in the development of cognitive impairments through the gut-brain-axis.

On this basis, we have explored, whether T1DM may alter [1] gut microbiome composition and diversity, [2] steroid levels in the colon,[3] gut permeability markers and [4] cognitive abilities and whether correlations among these aspects may occur in female STZ-induced rats.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (150-175 g at arrival, Charles Rivers Laboratories, Lecco) were used. Animals were housed in the animal care facility of the Dipartimento di Scienze Farmacologiche e Biomolecolari (Università degli studi di Milano, Milan, Italy). All animals were kept in individually ventilated cages (IVC), with food and tap water available ad libitum and under controlled temperature (21 ± 4°C), humidity (40-60%), room ventilation (12.5 air changes per h) and light cycles (12 – hour light/dark cycle; on 7 A.M./off 7 P.M.).

The rats were allowed to acclimate to new environment for 7 days before being randomly assigned to one of the experimental groups described below. Animal care and procedures were approved by our institutional animal use and care committee and followed Institutional guidelines that are following national (D.L. No. 26, March 4, 2014, G.U. No. 61 March 14, 2014) and international laws and policies (EEC Council Directive 2010/63, September 22, 2010: Guide for the Care and Use of Laboratory Animals, United States National Research Council, 2011). We have also followed the ARRIVE guidelines in the execution of animal experimental and in particular the ARRIVE essential 10.

Diabetic induction and characterization

Animals were randomly divided into two different experimental groups: i) non-diabetic animals (CTRL); ii) streptozotocin diabetic animals (STZ). To obtain diabetic condition, rats were injected with a single intraperitoneal (i.p.) of freshly prepared streptozotocin (60 mg/kg body weight; Sigma-Aldrich) in citrate buffer (0.09 M pH 4.8) as previously described (Pesaresi et al., 2010). Non-diabetic control animals received injections of citrate buffer alone. After 48h, diabetes was confirmed by tail vein blood glucose measurement using a commercial glucometer (Contour next, Ascensia Diabetes Care Italy, Milan, Italy) and only the rats with feeding blood glucose above 300 mg/dl were classified diabetic. Body weight was monitored every week.

Novel object recognition (NOR) test

The animals were tested in non-transparent open fields (100 cm in diameter, 35 cm high, with the floor divided into painted 16-cm squares). After 1 hour of adaptation session, the animals were allowed to explore two identical objects for 5 min (trial session-encoding phase). In the retention trial conducted one hour later (testing session), one of the objects presented previously was replaced by a novel object. Rats were returned to the open field and the duration of exploration of each object (i.e., sitting near the objects, sniffing, or touching them) was manually measured during the 5 min test. The task was performed in an isolated room under low lights conditions, in the absence of a direct overhead lighting. The NOR index was calculated according to the following formula: time of novel object exploration divided by time of novel plus familiar object exploration, multiplied by 100. In this experiment, after 4 weeks from STZ injection the NOR test was performed in both CTRL and STZ rats in the diestrous phase of estrous cycle to explore the possible negative effect of diabetic condition on the cognition and memory abilities. Moreover, NOR test was performed on all animals in the late morning, time when they do not have a peak of corticosterone, in order to minimize its influence on behavioral performance.

Estrous cycle analysis

Estrous cycle patterns were evaluated using vaginal smear method. Vaginal smears were collected once a day, at the same time (9.00 A.M. – 10.00 A.M.) for 7 consecutive days. The smear slides were analyzed under light microscopy to determinate the cell types observed in the vaginal smear and then the four different phases: proestrous, estrous, metaestrous, and diestrous. Female rats were sacrificed 1 month after the STZ or vehicle injection at the diestrus phase.

Sample collection

After one month from the determination of hyperglycaemic status, both CTRL and STZ were individually placed in an induction chamber with 2% isoflurane (ISO VET, La Zootecnica, Milan, Italy) until the loss of the righting reflex and hippocampus,

cerebral cortex and longitudinal sections of the colon were harvested, immediately frozen in liquid nitrogen and stored at −80°C until the analyses. Fecal samples were instead collected before anesthesia, and then immediately frozen in liquid nitrogen, transported to the laboratory, and stored at −80°C. Fecal samples were instead collected before anesthesia, and then immediately frozen in liquid nitrogen, transported to the laboratory, and stored at −80◦C. Also, blood samples were collected in tubes with EDTA 0.25 M and centrifugated at 2500 g for 15 min at 4°C to obtain plasma.

16S next-generation sequencing

16S rRNA analysis was performed using 16A-V4 as the target region (Klindworth et al. 2013). 16S rRNA gene sequencing was performed using Illumina technology at the DNA sequencing facility of GalSeq Srl (www.galseq.com). Following sequencing, low-quality reads were removed and raw fastq reads were subsequently inspected using the Illumina quality tested using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/).
When required, adaptor sequences were removed using Cutadapt (Martin 2011). The filtered fastq reads were processed using QIIME2 (Bolyen et al. 2019).

Liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS)

For the quantitative analysis of different neuroactive steroids, the hippocampus, cerebral cortex, colon, and plasma were extracted and purified as previously described by *Giatti et al*. (2021).
17β-Estradiol-2,3,4-$^{13}C_3$-17β-E (2ng/sample), progesterone-2,3,4,20,25-$^{13}C_5$ ($^{13}C_5$–PROG), (0.4 ng/sample) and pregnenolone-20,21-$^{13}C_2$-16,16 D_2($^{13}C_2D_2$– PREG) (10ng/sample), were used as internal standards. Neuroactive steroids levels were assessed based on calibration curves freshly prepared and extracted (Giatti et al., 2021). Briefly, the samples were spiked with labelled internal standards, and homogenized in 2 ml MeOH/acetic acid (99:1 v/v) using a tissue lyser (Qiagen,

Milan, Italy) and plasma samples were diluted in ACN. After an overnight extraction at 4 °C, the organic phase of each sample was evaporated to dryness, resuspended in MeOH/H_2O 1:9 (v/v) and purified using C18 SPE cartridges (HyperSep C18 SPE Columns 500mg 3ml; Microcolumn, Milano, Italy). The organic residues were resuspended with 3 ml MeOH/H_2O (10:90 v/v) and passed through SPE cartridges, previously activated with MeOH (5 ml) and MeOH/H2O 1:9 v/v (5 ml). The steroids were eluted in MeOH, concentrated, and transferred in autosampler vials before liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. The analysis was conducted by liquid chromatography (LC) supplied by Surveyor liquid chromatography (LC) Pump Plus and Surveyor Autosampler Plus (Thermo Fisher Scientific MA, USA) connected with a linear ion trap - mass spectrometer LTQ (Thermo Fisher Scientific MA, USA), operated in positive atmospheric pressure chemical ionization (APCI+) mode. The chromatographic separation was achieved with a Hypersil Gold column C18 (100 × 2.1 mm, 3 µm; Thermo Fisher Scientific MA, USA) maintained at 40 °C. The mobile phases consisted of 0.1% formic acid in H_2O (phase A) and 0.1% formic acid in MeOH (phase B). Gradient elution was as follows: 0–1.50 min 70% A; 1.50–2.00 min 55% A; 2.00–3.00 min 55% A, 3.00–35.00 min linear gradient to 36% A; 35.00–40.00 min 25% A; 41.00–45.00 min 1% A; 45.00–45.20 min 70% A and 45.40–55.00 min equilibrate with 70% A. 25 µl sample was injected at a flow rate of 300 µL/min. The divert valve was set at 0–8 min to waste, 8–45 min to source and 45–55 min to waste. The injector needle was washed with MeOH/H_2O 1:1 (v/v). LC-MS/MS data were acquired and processed using software Excalibur® release 2.0 SR2 (Thermo Fisher Scientific MA, USA).

Quantitative analysis was performed based on calibration curves prepared daily and analyzed as previously described (Caruso et al., 2013b). Briefly, blank samples [6% albumin in phosphate-buffered saline (PBS) or cortex homogenate (obtained from the control rats) were spiked with $^{13}C_3$-17β-E (2 ng/sample), C_{13}-PROG (0.4 ng/sample) and C_{13}-PREG (10 ng/sample), as internal standards. Increasing amounts (0.05–5 ng/sample) of each steroid were added. Calibration curves were

extracted and analyzed as described for the experimental samples. Positive atmospheric pressure chemical ionization (APCI+) experiments were performed with a linear ion trap MS (LTQ; ThermoElectron Co., San Jose, Calif., USA) using nitrogen as sheath, auxiliary and sweep gas. The instrument was equipped with a Surveyor LC Pump Plus and a Surveyor Autosampler Plus (ThermoElectron Co.). The MS was employed in tandem mode (MS/MS) using helium as collision gas. The LC mobile phases have been previously described (Caruso et al., 2013b). The Hypersil Gold column (100 × 3 mm, 3 ¬m; ThermoElectron Co.) was maintained at 40° C. Peaks of the LC-MS/MS were evaluated using a Dell workstation by means of the software Excalibur®, release 2.0 SR2 (ThermoElectron Co.). The samples were analyzed using the transitions previously reported (Caruso et al., 2013b).

Real time polymerase chain reaction (RT-PCR)

RNA was extracted from snap-frozen cerebral cortex, hippocampus and colon using Directzol ™ MiniPrep kit (Zymo Research, Irvine, Calif., USA) following manufacturing protocol after homogenization with EUROGOLD Trifast (Euroclone, Milano, Italy) in Tissue Lyser (Qjagen, Italy). The quantification of RNA was performed by NanoDrop™ 2000 (ThermoFisher scientific, Milano, Italy). Gene expression was assessed by TaqMan quantitative real-time PCR using a CFX96 real-time system (Bio-Rad Laboratories, Segrate, Italy). Samples run in 96-well formats in duplicate as multiplexed reactions with a normalizing internal control, 36B4 (Eurofins MWG-Operon, Milano, Italy) using Luna Universal One-Step RT-qPCR Kit (New England BioLabs inc., Ipswich, MA). Eurofins MWG Operon: GABA-A α1 fwd: GAGAGTCAGTACCAGCAAGAAC rev: AGAACACGAAGGCATAGCAC; GABA-A α3 fwd: TTCACTAGAATCTTGGATCGGC rev: TCTGACACAGGGCCAAAAC; GABA-A α5 fwd: GATCGGGTACTTTGTCATCCAG rev: TGATGCTGAGGGTTGTCATG; GABA-A β2 fwd: CTGGATGAACAAAACTGCACG rev: ACAATGGAGAACTGAGGAAGC. Whereas specific primers and probe mix for IL-6 (NM_012589.1), IL-1β (NM_031512.2), TLR-4 (NM_019178.1) and TNF-α (NM_012675.3), GABA-A δ (Rn01517017_g1) GABA-A γ2 (Rn00788325_m1) and PR (Rn00575662_m1) were purchased from Life Technologies Italia (Monza, Italy) while zonulin-1 (ZO-1, Rn02116071_s1); claudin-

1 (Cldn-1, Rn00581740_m1), mucin-2 (Muc-2, Rn01498206_m1) and pregnane X receptor (PXR, Rn00583887_m1) were purchased from Applied Bio-systems, Thermo Fisher Scientific (Monza, Italy).

Western blotting

For Western blotting, the hippocampus was homogenized using the Tissue Lyser (Qiagen, Italy) in a cold lysis buffer (PBS without Ca2+ and Mg2+, EDTA 0.5 M pH 8, Igepal) supplemented with a protease cocktail inhibitor (Roche Diagnostic spa, Monza, Italy), then homogenates were centrifugated at 2000 rpm for 5 min at 4° C to remove particulate matter The protein content of hippocampus and cerebral cortex lysates was quantified using a Bradford Assay (Bio-Rad, Segrate, Italy). Then, samples containing equal amounts of protein were heated to 100° C for 5 min, run on polyacrylamide gel (Bio-Rad, Segratexz, Italy) and then transferred to nitrocellulose membranes. For immunoblot detection, membranes were cut and then blocked on an orbital shaker for 1 h at room temperature in 10% non-fat dry milk or 5% bovine serum albumin (BSA). Successively, each membrane was exposed to primary antibodies which are listed in Table 1. Primary antibody of Synaptophysin, was used at 1:1000 dilution in 5% BSA, while primary antibodies of synapsin and syntaxin in PBS-T 2.5 % non-fat dry milk, while GAPDH and VDAC, as the protein housekeeping, primary antibody was used at 1:10000 and 1:2000 dilution in PBS-T 2.5 % non-fat dry milk and BSA 5%, respectively. After overnight incubation at 4°C and extensive washing, the membranes were incubated with an anti-rabbit or anti-mouse horseradish peroxidase conjugated secondary antibody, according to the primary antibody (see Table 1). After washing, the protein bands were detected on membranes using the ECL method (Bio-Rad, Segrate, Italy). ECL signals were acquired with a ChemiDocTM XRS+ system (Bio-Rad, Segrate, Italy) and analyzed with Image LabTM software version 5.2.1 (Bio-Rad, Segrate, Italy). The mean control value within a single experiment was set to 100 and all the other values were expressed as a percentage.

Table 1: List of primary antibodies.

Antibody	Code	Host
Synaptophysin	Cell signaling - 5461S	Rabbit
Synapsin	Synaptic systems - 106001	Mouse
Syntaxin	Synaptic systems - 110011	Mouse
OXPHOS	ABCAM - AB10413	Mouse
SOD2	Sigma - PA001814	Rabbit
GAPDH	Santa Cruz - SC_25778	Rabbit
VDAC	ABCAM - AB15895	Rabbit

Thiobarbituric Acid Reactive Substances (TBARS)

Tissue and plasmatic thiobarbituric acid reactive substances (TBARS) were determined as an index of reactive oxygen species (ROS) production. We used the formation of TBARS during an acid-heating reaction, which is widely ad-opted as a sensitive method for the measurement of lipid peroxidation, as previously described (Ha and Endou, 1992), with modifications. Briefly, 10 µg of tissue analysed (i.e., hippocampus and cerebral cortex) were homogenized in 400µl of lysis buffer (TrisHCl 0.1 M, pH 7.4; EDTA 1.34 mM; glutathione 0.65 mM) using a TissueLyser II (Qiagen, Hilden, Germany). Then, 100 µl of homogenate was mixed with 600 µl of phosphoric acid 1% and 200µl of TBA 0.6%. Samples were then incubated at 95 C for 1 h and then cooled to RT, extracted with 1ml of n-butanol and then centrifuged at 3000 RPM at 4°C for 20 min. For plasma, we used 100 µl of plasma stored at -20°C with a mixture of antioxidant reagent, EDTA 1.34 mM and glutathione 0.65 mM following the same protocol except the homogenisation phase. The supernatant was measured fluorometrically at an excitation wavelength of 532 nm and an emission wavelength of 553 nm. Quantification was done using the standard curve with malondialdehyde following similar conditions.

Statistical analysis

LC–MS/MS, real-time PCR and behavioral analysis were analyzed by unpaired two-tailed Student's t-test, after checking for normal distribution with the Kolmogorov–Smirnov test. $p < 0.05$ was considered significant. Analyses were performed using Prism, version 7.0a (GraphPad Software Inc., San Diego, CA, USA). α-diversity was calculated using the Evenness, Shannon, Faith, and Observed Features metrics using the Wilcoxon rank-sum test; β-diversity was calculated by Bray Curtis, Unweighted and Weighted Unifrac; beta statistical analyses were performed using Anosim and Permanova. Multiple tests were controlled by the Benjamini-Hochberg procedure; tests with q-values < 0.1 were considered statistically significant. Taxonomy was identified using SILVA as the reference database (Quast et al. 2013). Linear regression analysis and Pearson's correlation coefficient were computed to assess the potential relationship between 2 different variables.

RESULTS

Novel object recognition performance of female rats after one month of type 1 diabetes and in control animals

Novel object recognition (NOR) test was used to evaluate cognition abilities in an experimental model of T1DM versus control. As showed in *figure 1*, one month of diabetes mellitus induced by STZ treatment in female rats induced, at the diestrus phase, a significant decrease in the NOR index compared to the control (CTRL). Thus, type 1 diabetes mellitus (T1DM) significantly affects cognitive function in female animals.

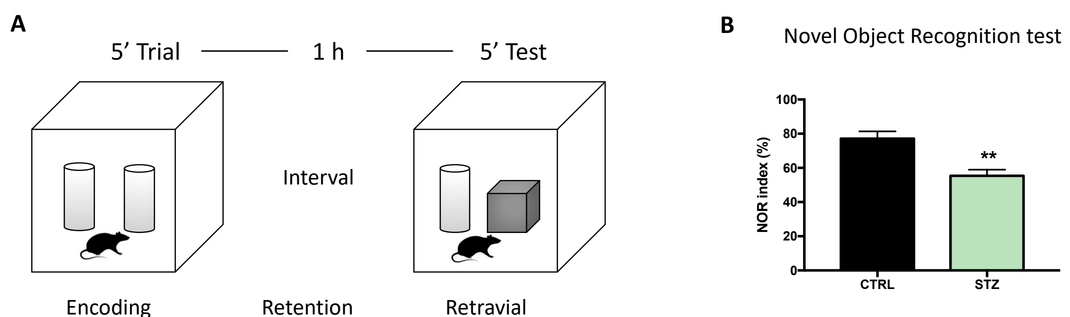

FIGURE 1: Effect of one month of diabetes on novel object recognition (NOR) performance. NOR test was carried out 4 weeks after STZ injection in non-diabetic (CTRL; n=7) and diabetic (STZ; n=7) female animals. Panel **A**: schematic picture of the novel object recognition test. Panel **B**. NOR index evaluated at the end of the experiment (4 weeks). The columns represent the mean± SEM in non-diabetic (CTRL; n=7) and diabetic (STZ; n=7) female animals. Statistical analysis was performed by Unpaired two-tailed Student’s t-test. *p < 0.05.

Neuroinflammation markers and synaptic proteins in hippocampus and cerebral cortex of female T1DM and control rats.

To assess the molecular modifications producing cognitive dysfunctions, we evaluated neuroinflammation and aberrant synaptogenesis, important mechanisms involved in the development of diabetic encephalopathy and cognitive impairment, firstly in hippocampus.

Therefore, we have analyzed the gene expression of important cytokines in our experimental model. As shown in *figure 2*, our data reveal that the relative mRNA expression of interleukin (IL)-1β (panel **A**) and IL-6 (panel **B**) was significantly increased by DM in the hippocampus of STZ female rats. No statistical difference

was observed in the expression of two other inflammatory cytokines considered, such as TNFα and TLR4 (panel **C**-**D**). The expression of synaptic proteins was also affected by one-month of diabetes mellitus in the hippocampus of STZ female rats. Indeed, as reported in *figure 2*, synaptophysin (panel **E**), synapsin (panel **F**) and syntaxin (panel **G**) were significantly decreased in diabetic animals compared to the control group.

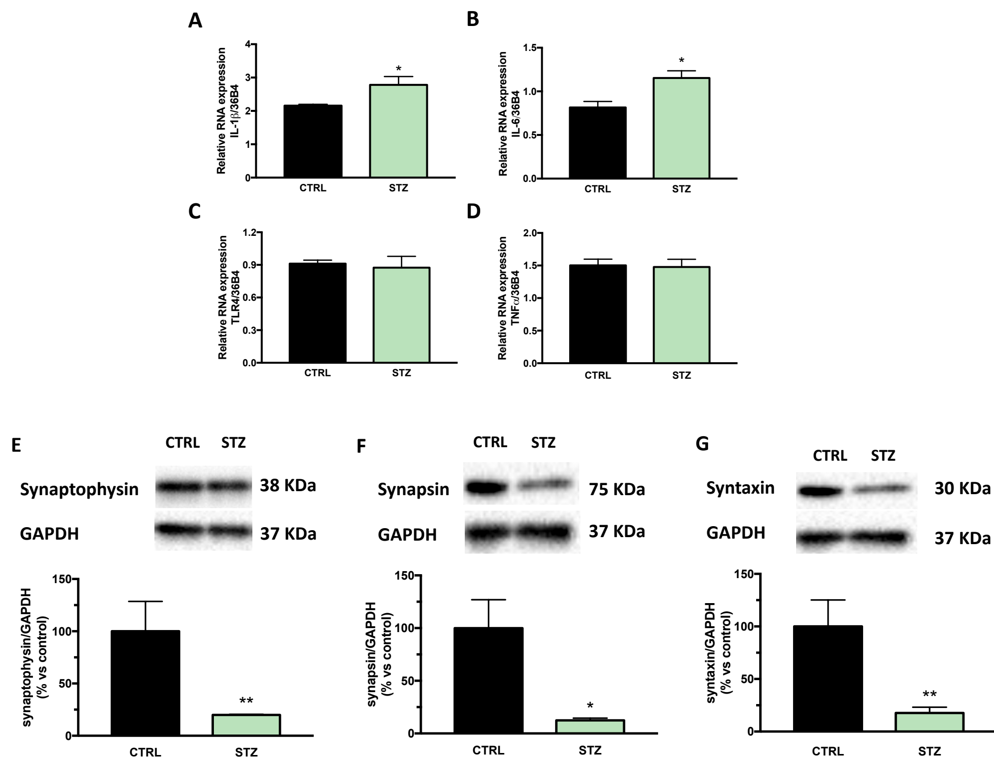

FIGURE 2: Effect of one month of diabetes on the gene expression of neuroinflammation markers and the protein content of synaptic proteins in hippocampus of female rats. IL-1β (panel **A**), IL-6 (panel **B**), TLR4 (panel **C**) and TNFα (panel **D**). The columns represent the mean ± SEM after normalization with 36B4 in non-diabetic (CTRL; n=7) and diabetic (STZ; n=7) female animals. Synaptophysin (panel **E**), synapsin (panel **F**) and syntaxin (panel **G**). The protein levels were detected by Western blotting in non-diabetic (CTRL; n=7) and diabetic (STZ; n=7) female animals. The columns represent the mean ± SEM after normalization with GAPDH. Statistical analysis is performed by Unpaired Student's t-test. *p < 0.05 and ** p< 0.01.

These effects seem to be brain specific area. Indeed, analysis performed in the cerebral cortex (*figure 3*) demonstrated that the relative mRNA expression of IL-1β (panel **A**) and IL-6 (panel **B**), as well as the expression of synaptic protein such as synaptophysin (panel **E**) and syntaxin (panel **G**) were unaffected by diabetes mellitus. In contrast to what was observed in the hippocampus (*figure 2* panel **C** and **D**) the relative mRNA expression of TLR4 (panel **C**) and TNF-α (panel **D**) was significantly increased by short term diabetes. In addition, at variance to what reported in the hippocampus (*figure 2*, panel **F**), the expression of synapsin in cerebral cortex of STZ-female animals (*figure 3*, panel **F**) was also significantly increased by the pathology.

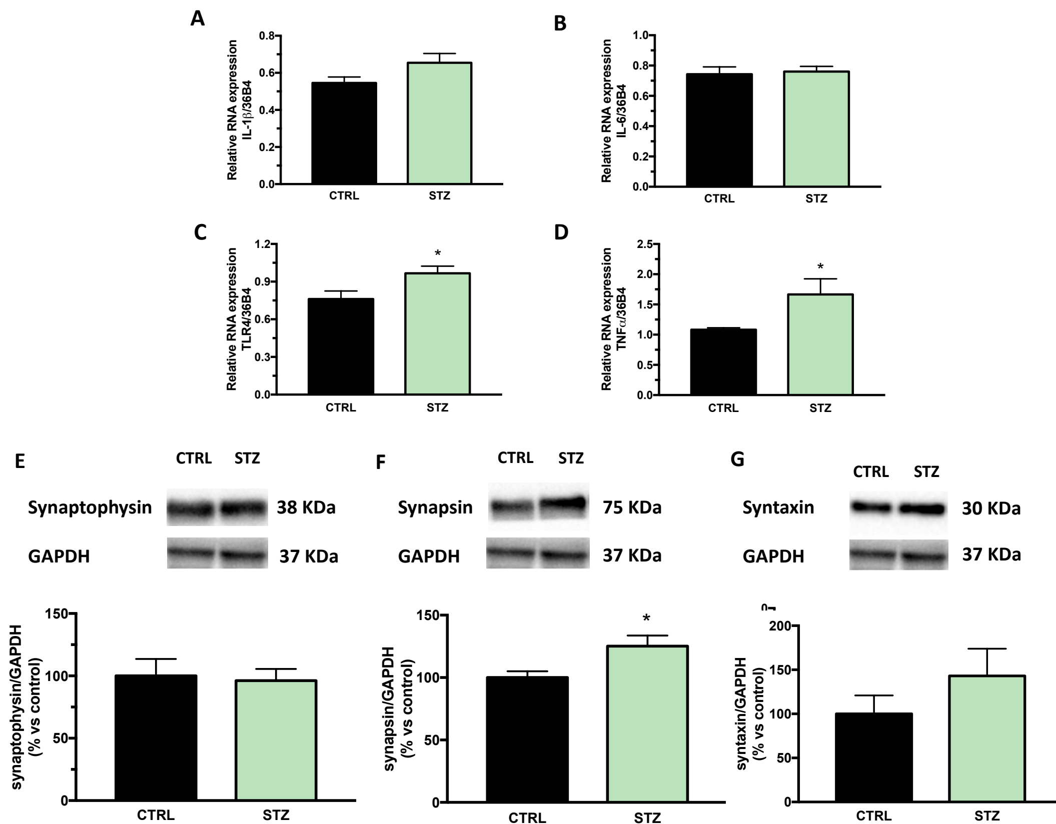

FIGURE 3: Effect of one month of diabetes on the gene expression of neuroinflammation markers and on protein content of synaptic proteins in the cerebral cortex of female rats. IL-1β (panel **A**), IL-6 (panel **B**), TLR4 (panel **C**) and TNFα (panel **D**). The columns represent the mean ± SEM after normalization with 36B4 in non-diabetic (CTRL; n=7) and diabetic (STZ; n=7) female animals. Synaptophysin (panel **E**), synapsin (panel **F**) and syntaxin (panel **G**). The protein levels were detected by Western Blotting in non-diabetic (CTRL; n=7) and diabetic (STZ; n=7) female animals. The columns represent the mean ± SEM after normalization with GAPDH. Statistical analysis is performed by Unpaired Student's t-test. *$p < 0.05$.

Oxidative stress in the hippocampus, cerebral cortex, and plasma of female T1DM and control rats.

We assessed the levels of TBARS in our experimental model, as index of reactive oxygen species production. As reported in *figure 4*, the TBARS levels were significantly increased in plasma (panel **C**) and in cerebral cortex (panel **B**), but not in hippocampus (panel **A**) of STZ-female animals, suggesting oxidative stress in periphery and a specific effect depending on the brain areas considered. As reported in panel **D**, protein levels of superoxide dismutase 2 (SOD2) were upregulated in the hippocampus but significantly decreased in the cerebral cortex of STZ-female animals by diabetic condition (panel **E**).

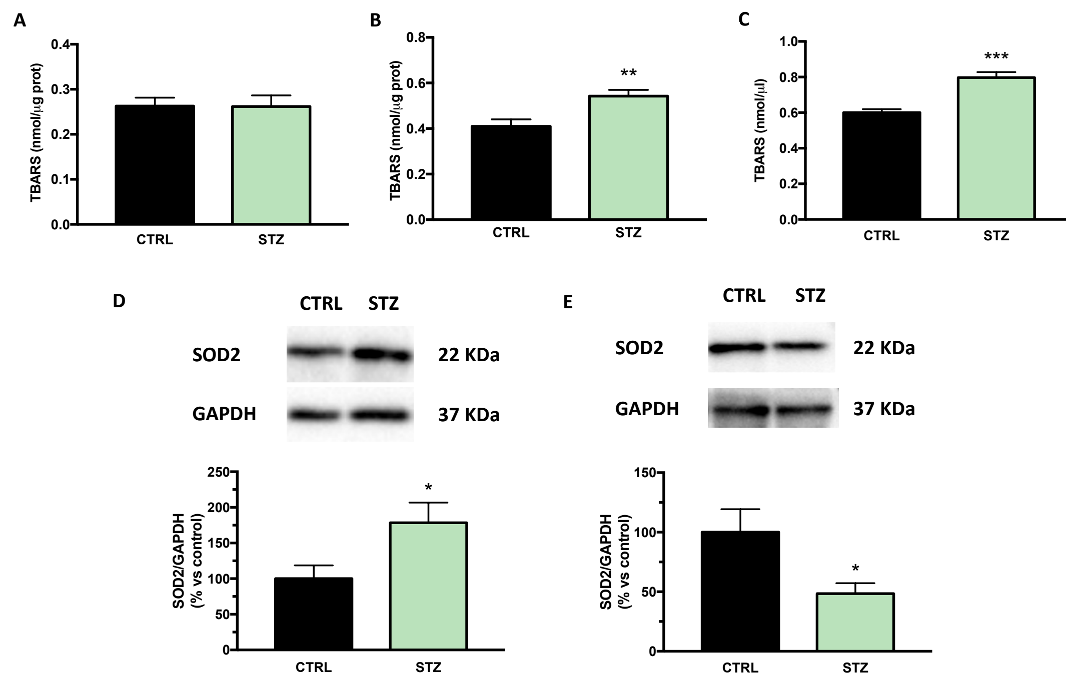

FIGURE 4: Effect of one month of diabetes on molecules involved in oxidative stress in hippocampus, cerebral cortex, and plasma. Thiobarbituric acid reactive substance (TBARS) in the hippocampus (panel **A**), in cerebral cortex (panel **B**) and in plasma (panel **C**). The columns represent the mean ± SEM. The protein levels of superoxide dismutase 2 (SOD2) were detected by Western Blotting in hippocampus (panel **D**) and cerebral cortex (panel **E**) of non-diabetic (CTRL; n=7) and diabetic (STZ; n=7) female animals. The columns represent the mean ± SEM after normalization with GAPDH in non-diabetic (CTRL; n=7) and diabetic (STZ; n=7) female animals. Statistical analysis is performed by Unpaired Student's t-test. * $p < 0.05$ ** $p < 0.01$. *** $p < 0.001$.

Mitochondrial functionality in female T1DM and control rats.

Mitochondrial functionality has been assessed by detecting OXPHOS levels. As shown in *figure 5* panel **A**, the protein content of different subunits belonging to respiratory chain NDUFA8B (complex I), SDHB (complex II), UQCRC2 (complex III), mt-COX2 (complex IV), and ATP5A (complex V) was unmodified in the hippocampus. On the contrary, except for complex II and complex III, complexes I, IV and V were significantly decreased in the cerebral cortex of female T1DM rats (panel **B**).

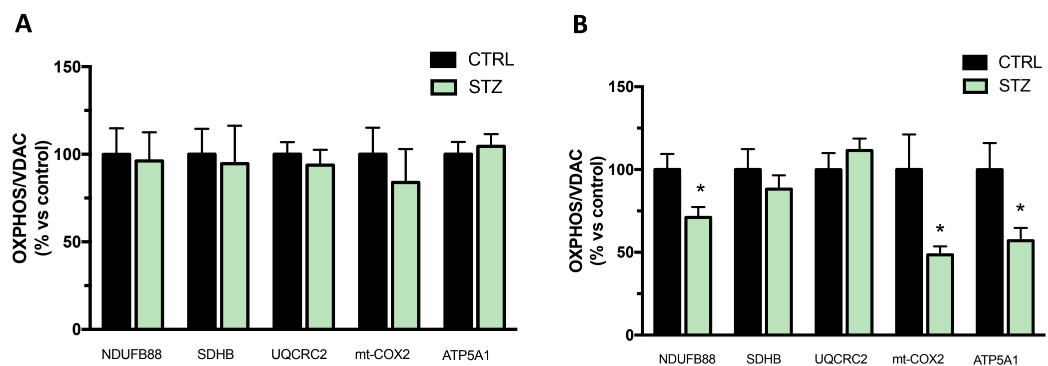

FIGURE 5: Effect of one month of diabetes on mitochondrial functionality in hippocampus and cerebral cortex. Protein contents of NDUFA8B (complex I), SDHB, (complex II), UQCRC2 (complex III), mt-COX2 (complex IV) and ATP5A1 (complex V) were detected by Western Blotting in hippocampus (panel **A**) and cerebral cortex (panel **B**) of control non-diabetic (CTRL) and diabetic (STZ) female rats. The columns represent the mean ± SEM after normalization with VDAC in non-diabetic (CTRL; n=7) and diabetic (STZ; n=7) female animals. Statistical analysis is performed by Unpaired Student's t-test. * $p < 0.05$.

Neuroactive steroid levels in the hippocampus, cerebral cortex, and plasma of female T1DM and control rats.

The levels of neuroactive steroids were assessed by LC-MS/MS in the hippocampus (panel **A**), cerebral cortex (panel **B**) and plasma (panel **C**) of control and STZ female rats (*figure 6*). As reported in panel **A**, a significant increase in the PROG metabolites, DHP and ALLO was observed in the hippocampus of STZ-female rats vs CTRL group. The levels of the other steroids assessed were not significantly modified by diabetic condition. The effect of diabetes mellitus was different in the cerebral cortex. Indeed, as reported in panel **B**, only a significant increase in the levels of ISOALLO, another PROG metabolite, was observed. Analysis of steroids in plasma showed a different pattern of effect in comparison to what observed in the brain areas here considered. Indeed, as reported in panel **C**, the levels of PREG and of its direct metabolite, PROG, as well as those of PROG metabolites, DHP and ALLO, were reduced in STZ female rats in comparison to control animals.

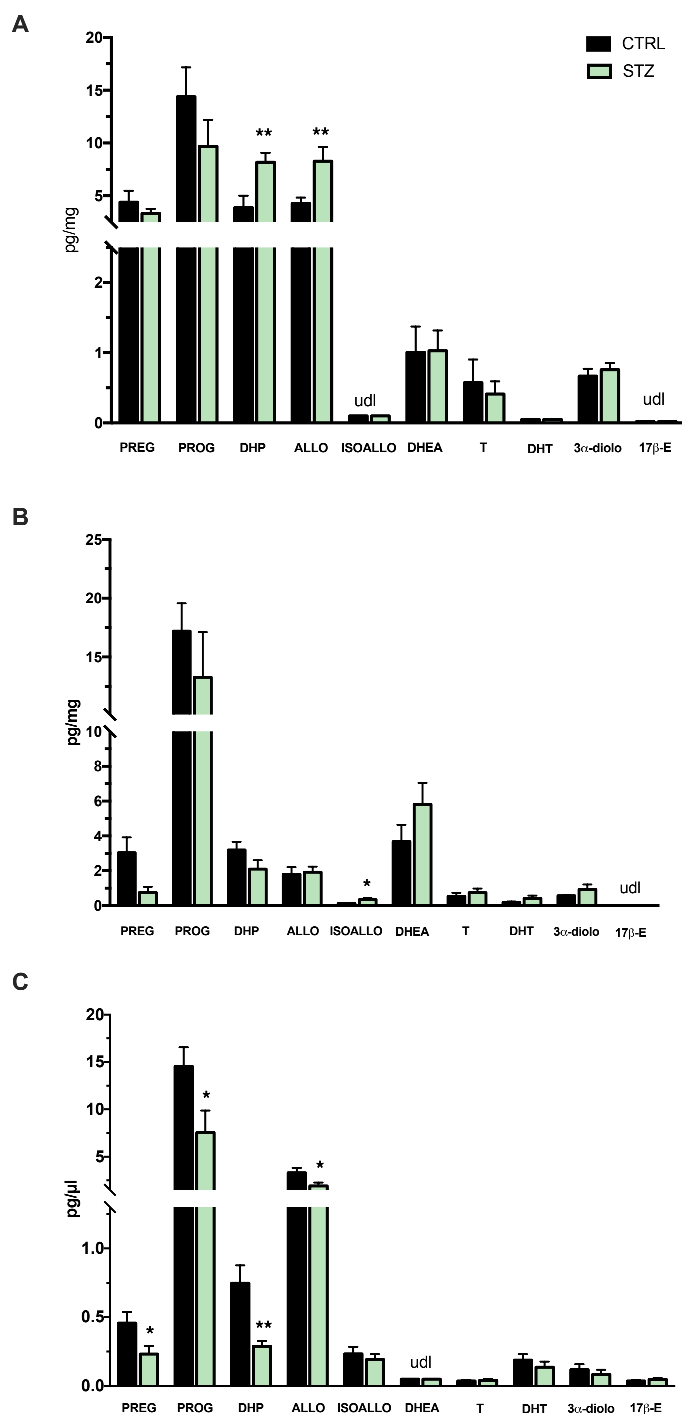

FIGURE 6: Effect of one month of diabetes on the level of neuroactive steroids in the hippocampus, cerebral cortex and plasma of female non-diabetic (CTRL; n=7) and diabetic (STZ; n=7) rats. Levels of neuroactive steroids in hippocampus (panel **A**), cerebral cortex (panel **B**) and in plasma (panel **C**). Data are expressed as pg/mg ± SEM in case of brain areas, and pg/µl ± SEM in case of plasma. u.d.l. = under detection limit. Detection limits were 0.02 pg/mg or pg/µl for testosterone (T) and 17β-Estradiol (17β-E), 0.05 pg/mg or pg/µl for pregnenolone (PREG), progesterone (PROG), 5α-androstane-3α,17β-diol (3α-diol), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT); 0.1 pg/mg or pg/µl for allopregnanolone (ALLO) and isoallopregnanolone (ISOALLO); 0.25 pg/mg or pg/µl for dihydroprogesterone (DHP). Statistical analysis was performed by unpaired two-tailed Student's t-test. $^{*}p < 0.05$. $^{**}p < 0.01$.

Gene expression of progesterone receptor and GABA-A receptor subunits in the hippocampus of female T1DM and control rats.

DHP and ALLO interact with different receptors (i.e., DHP with progesterone receptor, PR, and ALLO with GABA-A receptor). Therefore, we have evaluated whether changes in the levels of DHP and ALLO observed in the hippocampus of diabetic female rats were associated with changes in the gene expression levels of their receptors. As shown in *figure 7*, panel **A**, the gene expression levels of PR were similar in STZ and control animals. Assessment of different GABA-A receptor subunits showed that the levels of $\gamma2$ subunit, but not those of $\alpha1$, $\alpha3$, $\alpha5$, $\beta2$ and δ subunits, were significantly decreased in the hippocampus of STZ vs CTRL female rats (panel **B**).

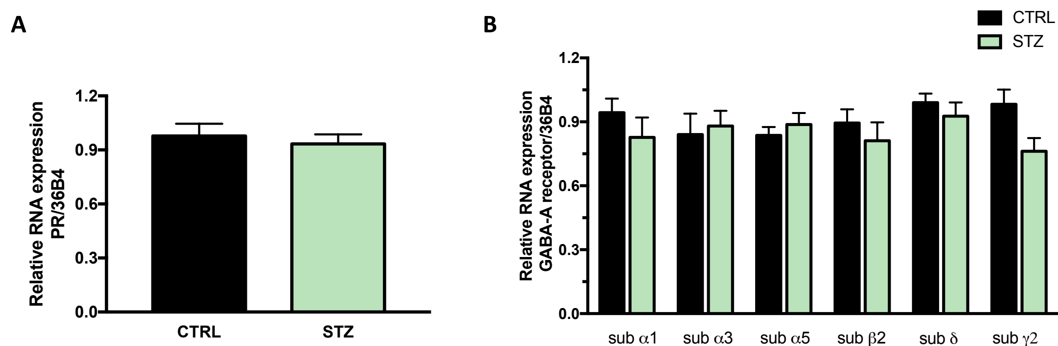

***FIGURE 7**: Effect of one month of diabetes on the gene expression of progesterone receptor (PR; panel **A**) and GABA-A receptor subunits, $\alpha1$,$\alpha3$, $\alpha5$, $\beta2$, δ, $\gamma2$ (panel **B**) in hippocampus of female rats. The columns represent the mean ± SEM after normalization with 36B4 in non-diabetic (CTRL; n=7) and diabetic (STZ; n=7) female animals. Statistical analysis is performed by Unpaired Student's t-test. *p < 0.05.*

T1DM modifies β-, but not α-diversity of gut microbiota in female STZ rats

In our study, the structure and composition of the gut microbiota were examined by 16S rRNA next-generation sequencing analysis. To identify changes in the intestinal microbiota richness due to T1DM, we analyzed α-diversity and β-diversity in stools collected 4 weeks after STZ induction. We have not observed a statistically significant change in α-diversity assessed by the *Shannon index*, *Evenness*, *Faith's phylogenetic distance* and observed ASVs. However, the analysis by Aitchison distance revealed statistically significant β-diversity between the two experimental groups using Adonis (p=0.002, R^2 = 0.30).

In the principal phyla observed in stool samples (i.e., *Firmicutes* CTRL: 58.73%; STZ: 61.26% and *Bacteroidetes* CTRL: 39.75%; STZ: 33.26%), we have not observed significant differences. However, several significant changes were observed in bacterial taxa composition. Significantly higher relative proportions of *Proteobacteria* and *Verrucomicrobiota* at the phylum level were found in diabetic rats (*Figure 8*). Accordingly, T1DM increased the bacterial class belonging to the *Gammaproteobacteria* class (including *Enterobacteriaceae* family), which were grouped in the phylum *Proteobacteria* (*Figure 8 and 9*, respectively). The analysis of taxonomy also highlighted that the increase of phylum *Verrucomicrobiota* were found at class (*Verrucomicrobiae*; *Figure 8*), at order (*Verrucomicrobiales*; *Figure 8*); at family (*Akkermansiaceae*; *Figure 9*) and at genus level (*Akkermansia*; *Figure 9).* Interestingly, as unweighted UniFrac distances suggested, others less predominant phyla, like *Desulfobacterota*, were affected (*Figure 8*). Indeed, in this phylum, also the order of *Desulfovibrionales* was significantly decreased in STZ female rats compared with control group (*Figure 8*). Moreover, we have observed significantly higher relative proportions of *Burkholderiales* (*Figure 8*), *Sutterellaceae* (*Figure 9*) and *Parasutterella* (*Figure 9*) at order, family, and genus levels in STZ rats compared with CTRL. Furthermore, T1DM significantly decreased bacterial taxa belonging to the genus *Enterorhabdus*, which were grouped in the order of *Coriobacteriales* (*Figure 9* and *8*, respectively) as well as to genus

Streptococcus, which were grouped in the family *Streptococcaceae* (*Figure 9*). These analyses have also revealed that the most representative genus of *Enterococcaceae* family (i.e., *Enterococcus*) as well as the *Enterobacteriaceae* family were significantly increased after 1 month of diabetes (*Figure 9).* In constrast, we have observed a significant decrease in *Anaerovoracaceae* and *Erysipelatoclostridiaceae* in STZ compared to CTRL rats (*Figure 9*). Similarly, we also detected an increase in the relative composition for multiple genera, such as *Blautia, Frisingicoccus, Holdemania, Anaerotroncus, Anaerostipes, A2* and *Paludicola* (*Figure 9*). In contrast, a significant decrease in *Erysipelotrichaceae, Candidatus Soleaferrea, Intestinimonas, Bilophila, Ruminococcaceae, Adlercreutzia, ASF356* and *Erysipelatoclostridium,* in STZ female rats was observed (*Figure 9*).

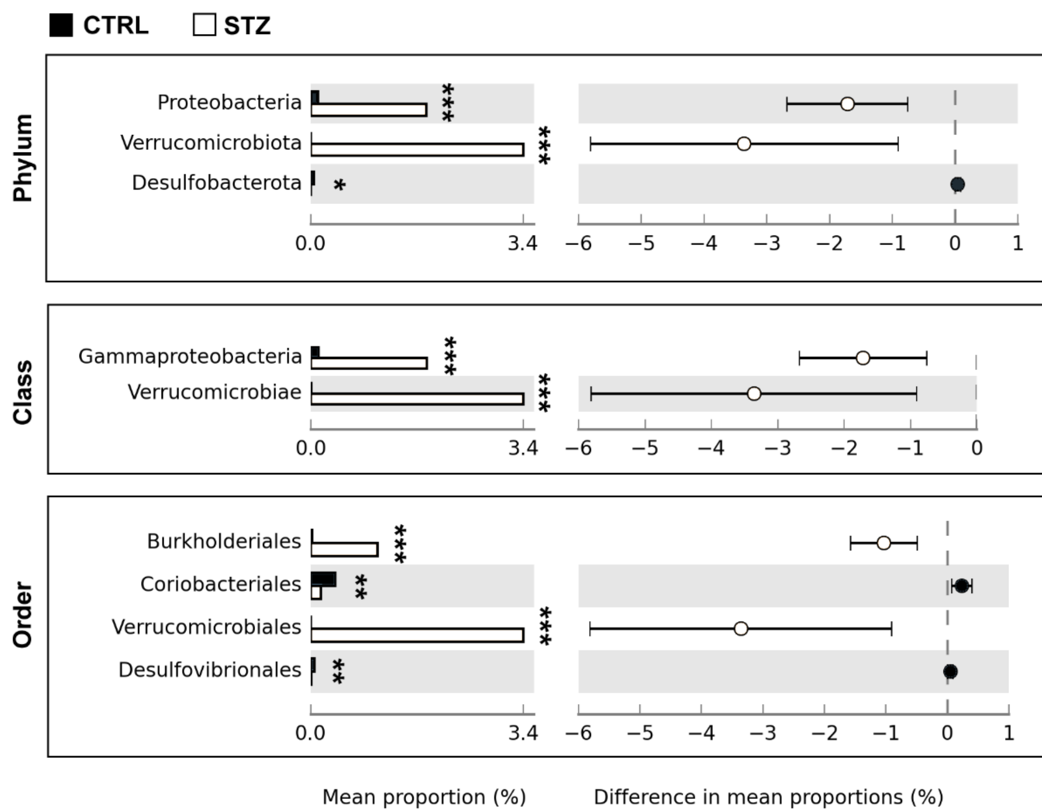

FIGURE 8: Mean proportions and difference in mean proportion are shown for significant different bacterial species for phylum, class, order and family taxonomy ranks in control (CTRL) and streptozotocin (STZ)-induced female rats. The black bars highlight the 95% confidence intervals of each analysis. Differential taxa were calculated using ANCOM-BC with Holm-Bonferroni correction, *p < 0.05, **p < 0.01, ***p < 0.001

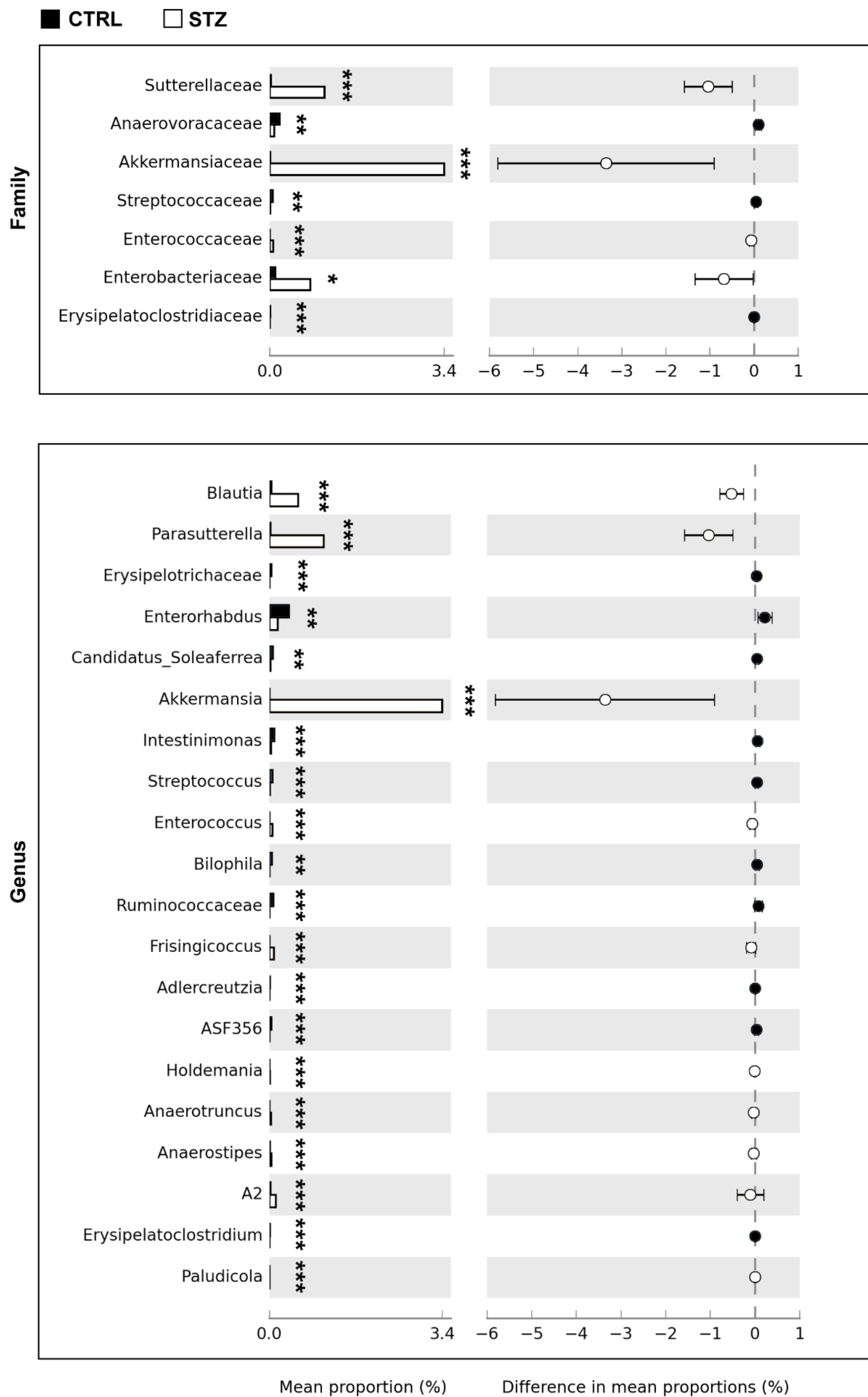

FIGURE 9: Mean proportions and difference in mean proportion are shown for significant different bacterial species for genus taxonomy ranks in control (CTRL) and streptozotocin (STZ)-induced female rats. The black bars highlight the 95% confidence intervals of each analysis. Differential taxa were calculated using ANCOM-BC with Holm-Bonferroni correction, *p < 0.05, **p < 0.01, ***p < 0.001.

Level of gut steroids after one month of diabetes in female T1DM and control rats

The levels of gut steroids were assessed by LC-MS/MS in the colon of CTRL and STZ female rats. As reported in *figure 10*, a significantly decreased of PREG (i.e., the first steroid synthesized from cholesterol) and significantly increased of ALLO, were observed after 1 month of diabetes in female rat colon. No statistical differences were observed in the levels of the other steroids analyzed.

Interestingly, we have also found a significant correlation between PREG and ALLO levels (*Figure 10*, panel **A**) and bacterial taxa modified by T1DM (*figure 8 and 9*). In particular, PREG showed a negative correlation with *Blautia* (panel **B**) but positive with *Streptococcus* (panel **C**). Moreover, ALLO presents a positive correlation with *Akkermansia* (panel **D**), *Gammaproteobacteria* (panel **E**), *Parasutterella* (panel **G**) and *Holdemania* (panel **K**) and negatively with *Enterorhabdus*, (panel **F**), *Anaerovoracaceae* (panel **H**), *Streptococcus* (panel **I**), *Erysipelotrichaceae* (panel **J**), *Candidatus Soleaferrea* (panel **L**) and *Ruminococcaceae* (panel **M**).

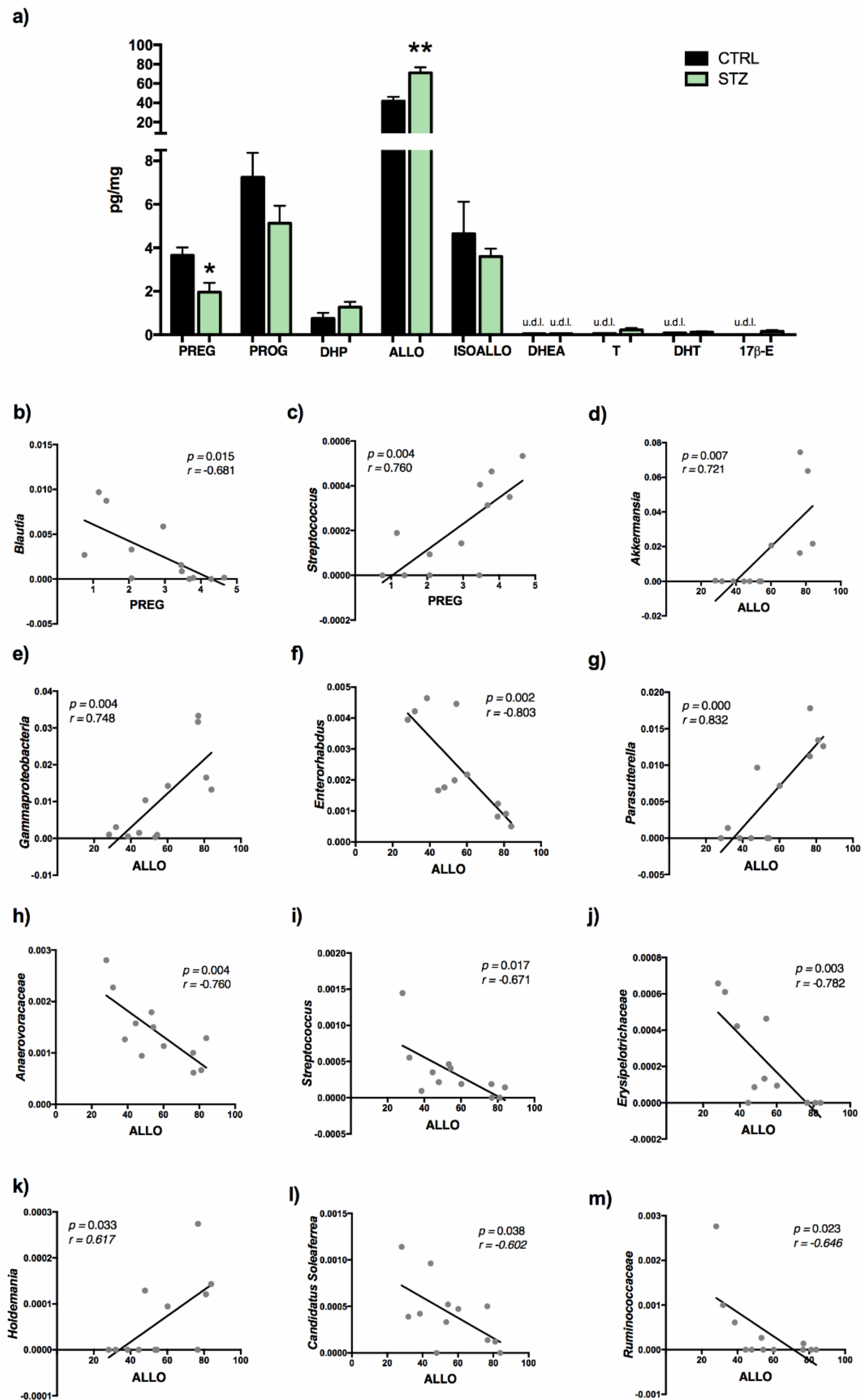

FIGURE 10: Effect of one month of diabetes on the levels of gut steroids in the colon of control (CTRL) and streptozotocin (STZ)-induced female rats (panel **A**). The unpaired Student's t-test was used for statistical analysis. $^*p < 0.05$, $^{**}p < 0.01$. Data are expressed as pg/mg ± SEM, u.d.l. = under detection limit. Detection limits were 0.02 pg/mg for testosterone (T) and 17β-estradiol (17β-E), 0.05 pg/mg for pregnenolone (PREG), progesterone (PROG), 5α-androstane-3α,17β-diol (3α-diol), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT); 0.1 pg/mg for allopregnanolone (ALLO) and isoallopregnanolone (ISOALLO); 0.25 pg/mg for dihydroprogesterone (DHP). Panels **B** to **M**: correlation of gut steroid levels with bacterial taxa. The p-value (*p*) and Pearson's correlation coefficient (*r*) are reported.

Gut permeability markers are affected in T1DM female rats

The intestinal surface barrier is one of the most important components of the innate immune system, but, as it known, diabetes can alter intestinal integrity. Indeed, as shown in *figure 11* panel **A**, the relative mRNA expression of tight junctions ZO-1 and Cldn-1, was significantly decreased by T1DM in female rats. Additionally, the gene expression of Muc-2, a gene known to be important for the mucous barrier that serves to protect the intestinal epithelium, was also significantly decreased in STZ compared to CTRL rats (*Figure 11,* panel **A**).

Also in this context, we have found several correlations among gut permeability markers, analyzed here, and gut microbiota populations altered by experimental T1DM (panel **B-Q**). In particular, ZO-1 was negatively correlated with *Akkermansia, Parasutterella, Gammaproteobacteria* and positively correlated with *Enterorhabdus.* Moreover, we also observed several correlations between tight junction Cldn-1 and bacterial taxa. Indeed, positive significant correlations (i.e., *Streptococcus, Anaerovoracaceae, Candidatus Soleaferrea)* and negative significant correlations (i.e., *Gammaproteobacteria, Parasutterella, Blautia, Holdemania* and *Enterococcaceae*) were identified*.* Furthermore, Muc-2 correlated positively with *Candidatus Soleaferrea* and *Enterorhabdus* and negatively with *Parasutterella* and *Holdemania.*

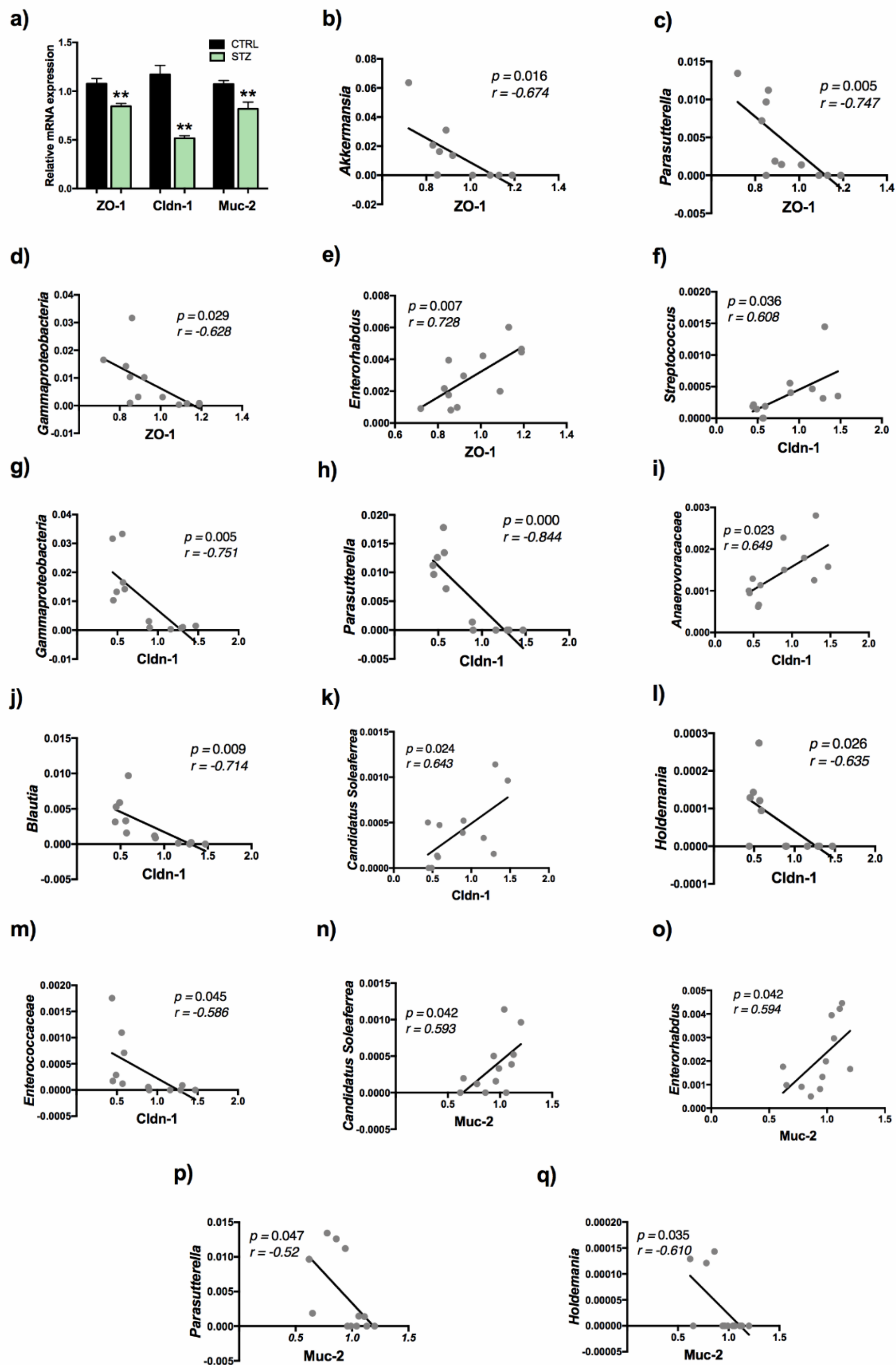

FIGURE 11: Effect of one month of diabetes on the gene expression of zonulin-1 (zo-1), claudin-1 (cldn-1) and mucin-2 (muc-2, panel **A**) in colon of control (CTRL) and streptozotocin (STZ)-induced female rats. The columns represent the mean ± SEM after normalization with 36B4. The unpaired Student's t-test was used for statistical analysis. $^{**}p < 0.01$. Panels **B**-**Q**: correlation of gut permeability markers with bacterial taxa. The p-value (p) and Pearson's correlation coefficient (r) are reported.

As shown in the previous paragraph (*figure 1*), diabetes after one month decreased NOR index of STZ group compared to CTRL group. Then, diabetes significantly affects hippocampal-dependent memory as well as induces alteration of gut microbiota composition (*figure 8 and 9*).

Linear regression analysis and Pearson's correlation coefficient were calculated to assess the possible relationships between gut microbiota composition and the performance in the cognitive test. Only the bacterial taxa that were significantly modified by T1DM were correlated with the NOR index. As reported in *figure 12*, a significant negative correlation between the NOR index and *Gammaproteobacteria* (panel **B**), *Parasutterella* (panel **C**), or *Blautia* (panel **D**) was observed.

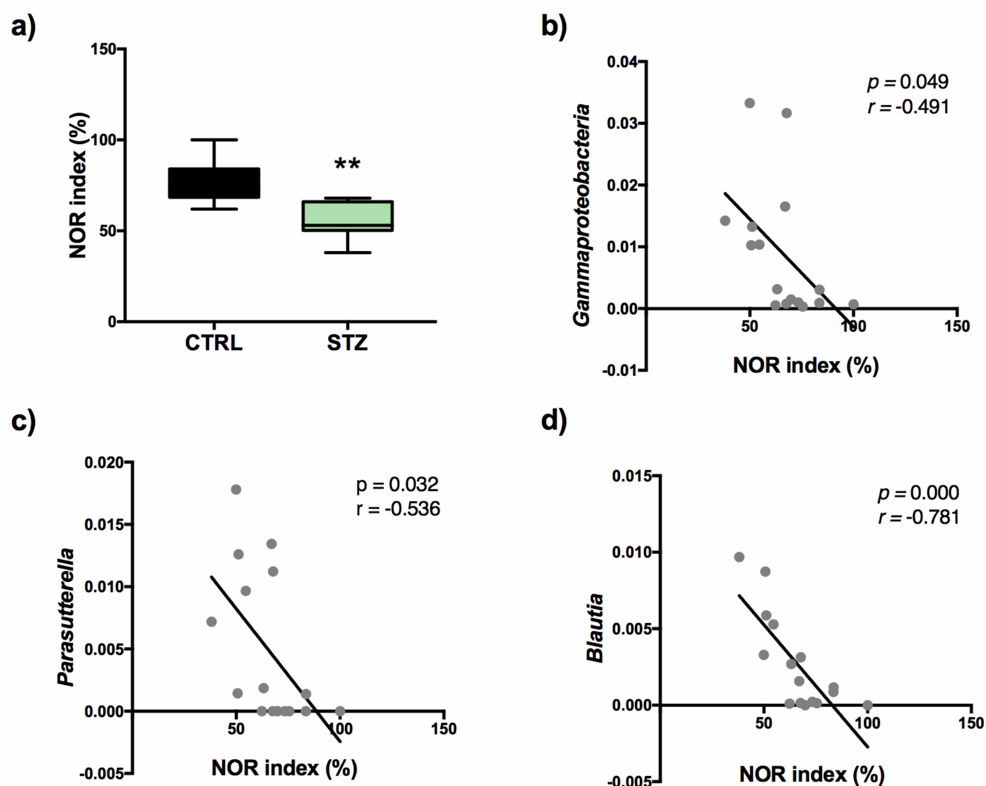

FIGURE 12: Novel object recognition (NOR) index control (CTRL) and streptozotocin (STZ)-induced female rats (panel **A**). The unpaired Student's t-test was used for statistical analysis. **p < 0.01 vs. CTRL. Panels **B** to **D** correlation of cognitive impairment with bacterial taxa. The p-value (p) and Pearson's correlation coefficient (r) are reported.

Gut steroids, behavioral impairment and gut permeability markers are related each other in experimental T1DM female rats

As reported above, the behavioral performance, gut steroids (i.e., PREG and ALLO) and gut permeability markers (i.e., ZO-1, Cldn-1 and Muc-2) correlated with gut microbiota populations altered in experimental STZ female rats. On this basis, we evaluated whether these parameters also correlated to each other. As reported in *figure 13*, linear regression and Person's correlation coefficient show that PREG levels (panel **A**) are positively correlated with Cldn-1 while ALLO (panel **B**) is linked negatively with this permeability marker. However, no significant correlations between these steroids and ZO-1 or Muc-2 were found (data not shown). Moreover, PREG levels (panel **C**), but not ALLO levels (data not shown) positively correlate with the NOR index. Interestingly, NOR index is also correlated with Cldn-1 (panel **D**), but not with ZO-1 and Muc-2 (data not shown).

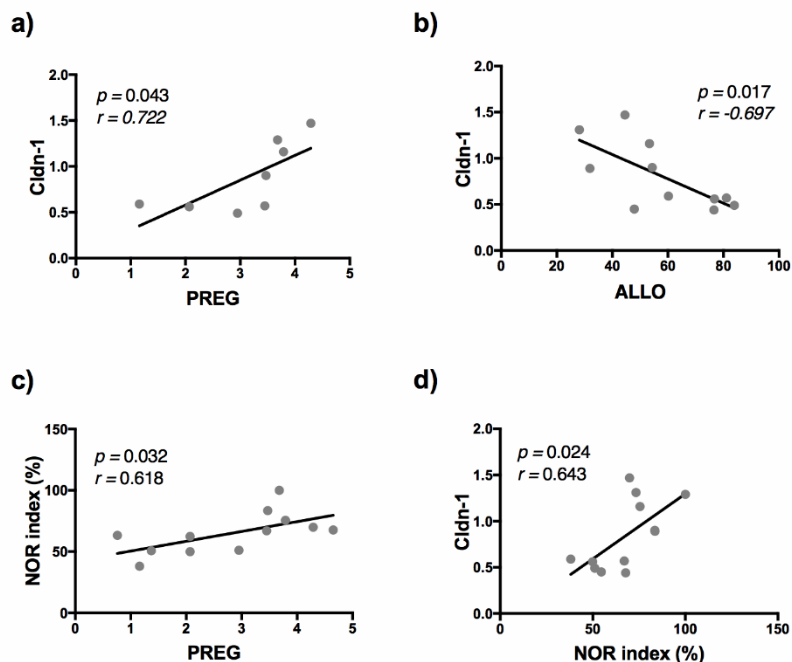

FIGURE 13: Correlation of pregnenolone (PREG) (panel **A**) and allopregnanolone (ALLO) (panel **B**) with claudin-1 (cldn-1), correlation of PREG with NOR index (panel **C**) and correlation of NOR index with cldn-1 (panel **D**). The p-value (p) and Pearson's correlation coefficient (r) are reported.

DISCUSSION

Diabetic encephalopathy (DE) is one of the severe microvascular complications of diabetes, characterized by impaired cognitive and memory functions and electrophysiological, neurochemical, and structural abnormalities (Cai et al., 2011). These alterations are associated with acute alterations in mental status due to poor metabolic control, decline in cognitive processes and increase risk of dementia, stroke, cerebrovascular and Alzheimer's disease and psychiatric disorders, such as depression, and eating disorders (Gispen and Biessels, 2000, Biessels et al., 2002, Jacobson et al., 2002, Biessels et al., 2008, Kodl and Seaquist, 2008).

Cognitive and memory impairments, which characterized DE, are associated with hippocampal dysfunction, but the mechanisms behind these impairments are not fully known. Data collected in literature, have demonstrated that the development of cognitive deficit seems to be the result of the concomitant presence of different processes such as oxidative stress, mitochondrial dysfunction, neuroinflammation and aberrant synaptogenesis in different brain areas of male rat models of type 1 DM (T1DM). Our previous studies have also demonstrated, in male streptozotocin (STZ)-treated rats, that also neuroactive steroids, important regulators of nervous functions, are involved in the development of diabetic encephalopathy since diabetic condition is able to affect the brain levels of these molecules (Romano et al., 2017; Romano et al., 2018).

Data obtained in this thesis indicate that one month of T1DM induced by STZ in female rats is able to decrease memory abilities, evaluated by the novel object recognition (NOR) test. This result is associated, in the hippocampus, with neuroinflammation (i.e., increase in the levels of IL-6 and IL-1β) and aberrant synaptogenesis (i.e., decrease in the levels of synaptophysin, synapsin and syntaxin) but not with oxidative stress, measured by TBARS levels, as confirmed by the lack of alteration in the mitochondrial functionality, measured by OXPHOS levels. These effects seem to be brain area specific. Indeed, levels of IL-1β and IL-6 as well as those of synaptophysin and syntaxin, are unaffected in female cerebral cortex. In contrast, an increase in TNFα, TLR4, and in synapsin levels as well as

oxidative stress and altered mitochondrial functionality have been observed in this brain area.

The different results obtained in the hippocampus and cerebral cortex may be related to the different impact of T1DM on the neuroactive steroid levels analyzed in both brain areas. Indeed, we observed that one month of pathology induced a significant increase of DHP and ALLO levels in the hippocampus of female rats, while in the cerebral cortex only an increase in ISOALLO levels was observed. This impact of T1DM is specific for these brain areas, since in plasma, levels of DHP and ALLO were decreased, while those of ISOALLO were unaffected. Furthermore, a significant decrease of PREG and PROG levels was reported. Thus, the neuroactive steroid levels in the two brain areas analyzed were differently affected by short-term diabetes and the change occurring in these brain areas do not reflect the effect of T1DM in the plasma levels. Brain region specificity as well as differences between brain and plasma steroids levels have also been demonstrated in physiological and other pathological condition (Melcangi et al., 2014; Caruso et al., 2013).

Therefore, the increased levels of DHP and ALLO in hippocampus but not in cerebral cortex and plasma could be explain as a possible protective effect of these molecules. Indeed, it is largely known that these metabolites of progesterone act as neuroprotective agents (Djebaili et al., 2004; Sayeed et al., 2006; Sayeed et al., 2009; Ciriza et al., 2006; Giatti et al., 2020) by interaction with progesterone receptor (PR), in case of DHP, or GABA-A receptor in case of ALLO (Melcangi et al., 2008; Belelli et al., 2005; Hsie et al., 2007; Guennon et al., 2015; Schumacher et al., 2014).

As reported here, the mRNA levels of PR are not affected in the hippocampus of female T1DM rats, while the gene expression of $\gamma 2$ subunit of GABA-A receptor were significantly decreased. Indeed, as previously reported, ALLO treatment is able to decrease the gene expression of this GABA-A subunit (Follesa et al., 2000; Biggio et al., 2006). Then, a possible hypothesis may link the increase in ALLO here reported in the hippocampus with the absence of oxidative stress and alteration

in mitochondrial functionality. Indeed, it has been demonstrated, in different neuropathological experimental models, ALLO treatment protect mitochondria and reduces oxidative stress Lejri et al., 2019; Cho et al., 2018; Lejri et al., 2017). For instance, in an experimental model of epilepsy, ALLO treatment increased the expression of SOD2. this, in turn reduces DNA fragmentation, oxidative damage, cell death and the production of ROS in the hippocampus. In addition, ALLO can increase mitochondrial oxygen consumption rate and ATP production in SH-SY5Y cells. Furthermore, after exposure to H_2O_2 alone or in combination with amyloid beta (Aβ) overproduction, ALLO was able to increase the reduced levels of ATP and decrease oxidative stress (Lejri et al., 2017). A further support of the hypothesis that ALLO may be protective against altered mitochondrial dysfunction and oxidative stress in the hippocampus is the finding that a decreased OXPHOS levels and increased TBARS occurred in the cerebral cortex where ALLO levels are unaffected.

As mentioned previously, the only data provided about the impact of one month of type 1 DM on cognitive function have been obtained in male animals. Data reported in literature indicate that in male animals the memory impairment induce by STZ injection is correlated with alteration of synaptic protein and neuroinflammatory pattern in the hippocampus. These observations are in agreement with what observed in female, even if also altered mitochondrial functionality (Romano et al., 2017; Hamed et al., 2017; Sadeghi et al., 2016) and oxidative stress were reported in male hippocampus (Nagayach et al., 2022; Wang et al., 2018; Rebai et al., 2017; Pereira et al., 2018,), suggesting that, in two sexes, there are different mechanisms which reduced memories abilities. This difference between two sexes seems to be specific for the hippocampus. Indeed, in agreement with what reported in male T1DM rats, oxidative stress (Masola et al., 2018; Minaz et al., 2018; Elahi et al., 2016; Romano et al., 2018) and mitochondrial dysfunction (Romano et al., 2018) were observed in the cerebral cortex of female diabetic rats. However, other small sex differences may also occur. For example, TBARS levels were increased in the female cerebral cortex, but not in males,

probably because males upregulate SOD2 (Romano et al., 2018), that is opposite as here described in females. Additionally, a significant decrease in complexes I, IV, and V was here observed in females, while in males only a decrease in complex IV was reported (Romano et al., 2018). Interestingly, also *McGovern and collaborators* have observed that the expression of three important proteins (i.e., NDUFA2, NDUFA7 and UQCR10) which belong to complex 1 and complex 3 respectively, is significantly altered by compounding factors that contribute to sex differences (McGovern et al., 2022). Moreover, in a model of chronic restraint stress, it has been highlighted that the activity of mitochondrial respiratory chain is altered by stress in a sex specific manner inducing for instance a reduced complex II hippocampal activity in males and increased in females (de Suoza Mota et al., 2017).

In terms of oxidative stress, the sex dimorphism reported in the hippocampus was not present in the periphery. Indeed, TBARS plasma levels increase in both females and males (Romano et al., 2017; Romano et al., 2018) . This may be imputable to different mechanisms in increasing oxidative stress in the brain and in blood circulation.

Our previous data reported that, the levels of neuroactive steroids are altered in the brain areas and in plasma of T1DM male rats. However, the impact of diabetes on these molecules. Indeed, in contrast to what here observed in female rats, in the male hippocampus (Romano et al., 2017) and cerebral cortex (Romano et al., 2018) we reported a decrease in the levels of PREG, PROG, ALLO, T, DHT and 3α-diol. In addition, in male hippocampus a decrease in the levels of ISOALLO was also reported (Romano et al., 2017). Like in case of female diabetic animals, also changes in plasma levels of male diabetic animals did not exactly reflect that observed in brain regions and show sex dimorphism. Indeed, in the plasma of male diabetic animals, a decrease in the levels of ISOALLO, T and 3a-diol was observed (Romano et al., 2017; Romano et al., 2018).

Altogether results obtained here about effect of one month of DM on brain, suggest that, in the female rat brain affected by T1DM, memory dysfunction can

be associated with aberrant synaptic function and neuroinflammation in the hippocampus. However, mitochondrial functionality and oxidative stress are not affected in this brain area, possibly due to the results of locally increased levels of DHP and ALLO. Additionally, these issues are specific for hippocampus since the cerebral cortex does not present a similar increase in steroid levels and consequently shows increased neuroinflammation and oxidative stress possibly due to dysfunctional mitochondria. Overall, these data indicate that in the female brain, after one month of DM induction, there is an attempt to counteract the effects of pathology specifically in hippocampus, that is probably not effective and thus produces memory deficits.

In the second part of this thesis, we focused our attention on the possible role of gut microbiota on the development of memory deficits in the female T1DM rats in the contest of gut brain axis.

The link between the pathogenesis of T1DM and gut microbiota was first investigated in an experimental model of DM, non-obese diabetic (NOD) mice (Suzuki, 1987), but later many studies are performed also in BioBreeding diabetes-prone (BB-DP) rats (Boerner & Sarvetnick 2011, Roesch et al. 2009, Yurkovetskiy et al. 2013). However, the influence of sex steroids in microbiota in T1DM is more recent (Markle et al., 2013), demonstrating the importance of microbial colonization in the modulation of host hormonal levels. Even thought, different experimental model of type 1 DM, including STZ-induced, show dysbiosis (Patterson *et al.* 2015, Gianchecchi & Fierabracci 2017, Fuhri Snethlage *et al.* 2021).

Our data demonstrated that, at variance to what observed in adult male STZ rats (Patterson et al. 2015), α-diversity is not compromised in females, suggesting that in this T1DM experimental model the decrease in species richness is more severe in male than in female rats. In this context, it is important to underline that sex-dependent effects on the gut microbiota in T1DM rodents have been observed (Pozzilli *et al.* 1993, Zhang et al. 2021). Moreover, as reported by taxonomical analysis, T1DM induces changes in the microbial composition. Particularly, in

female T1DM rats, we observed an increase in the abundance of *Proteobacteria, s*pecifically of the *Gammaproteobacteria* class (including *Enterobacteriaceae* family). Interestingly, *Gammaproteobacteria* are considered pro-inflammatory taxa (Liu *et al.* 2020), and the *Enterobacteriaceae* family includes inflammogenic enteric pathogens (Maes *et al.* 2008), which can translocate in the systemic circulation when the intestinal barrier is compromised (O'Malley *et al.* 2010). Accordingly, the increase in the abundance of *Proteobacteria*, which are intricately involved in dysbacteriosis observed in Inflammatory Bowel Disease (IBD) (Chen *et al.* 2018, Mukhopadhya *et al.* 2012), was also detected in male STZ rats (Patterson et al. 2015). The *Proteobacteria* phylum includes not only *Gammaproteobacteria* but also *Alphaproteobacteria* and *Betaproteobacteria*, which are generally enriched in patients with Crohn's disease (Kaakoush *et al.* 2012). Notably, *Burkholderiales* order (including genus *Parasutterella*), belonging to the *Betaproteobacteria* phylum, is also significantly enriched in female diabetic rats compared with controls, suggesting that the presence of a diabetic phenotype promotes the occurrence of a pro-inflammatory microenvironment. Furthermore, we also observed in female diabetic rats a significant increase in *Blautia* that *play* a role in metabolic and inflammatory diseases, as well as in biotransformation, even if the causal relationship between *Blautia* abundance and disease is still unclear (Liu *et al.* 2021). Interestingly, the same increase was observed in patients affected by T1DM (Kostic *et al.* 2015). In this study, *Blautia*, *Gammaproteobacteria* and *Parasutterella* are negatively correlated with the NOR index, suggesting an important role of these taxa in cognitive function in this early stage (i.e., one month of T1DM). In addition, *Blautia* correlates with PREG, the first precursor of steroid molecules, whereas this genus was already associated with gut steroids (Diviccaro et al., 2022), suggesting a crucial involvement in the steroid pathway.

In agreement, as here observed, the pro-inflammatory profile of taxa affected, is associated with a dysfunction of intestinal permeability. Indeed, we have observed that both tight junctions and Muc-2 are significantly altered in female T1DM rats. Notably, the integrity of intestinal permeability is guaranteed by the proteins

involved in the tight junctions between epithelial cells, which are able to regulate gut permeability, such as the Cldn-1 and ZO-1 protein families (Panwar *et al.* 2021) and by the first barrier on the surface of the GI tract (i.e., the mucus layer) of whom Muc-2 is the most important component (Vancamelbeke *et al.* 2017, Yao *et al.* 2021). It is thought that intestinal permeability is an initiator or accelerator of T1DM (Odenwald & Turner 2013, Buckner & Greenbaum 2017). Indeed, studies carried to T1DM patients (Pellegrini et al., 2017) reported that the disruption of intestinal permeability, known as the leaky gut syndrome, is related to dysbiosis. In fact, studies in humans and animal models indicate that a disrupted gut microbiome contributes to T1DM pathogenesis. In this contest, although *Akkermansia* is generally known for its beneficial properties (Kim *et al.* 2021, Shin *et al.* 2014, Ou *et al.* 2020), an overabundance of this species, as here observed in female T1DM rats, is not always advantageous (Arthur *et al.* 2012, Chassaing *et al.* 2015). In this study we also reported a significant positive correlation between *Akkermansia* and ALLO, a steroid that is well-known to have anti-inflammatory effects (He *et al.* 2004, Fujii *et al.* 2021, Yilmaz *et al.* 2019, Diviccaro *et al.* 2021b). Indeed, this steroid is able to bind the GABA-A receptors (Belelli & Lambert 2005, Gunn *et al.* 2014), and ligands of this neurotransmitter receptor, have been proposed as therapeutic tools in major peripheral inflammatory disorders, such as IBD (Dudley *et al.* 2011). Therefore, we hypothesize, that the strong increase in the gut levels of this PROG metabolite is a protective response of the female host to cope with inflammation in the microbial environment. At variance to ALLO levels, we here reported that the levels of PREG were decreased in female diabetic gut. Very little is known about the local role of this cholesterol metabolite (i.e., PREG) in the GI tract. However, it is important to highlight that an orphan nuclear receptor, such as the PXR, is activated by PREG (Kliewer *et al.* 1998). This nuclear receptor is highly expressed in the intestine (Kliewer et al. 1998), where it promotes anti-inflammatory responses (Shah *et al.* 2007). Interestingly, we here reported that the low PREG levels observed in the GI of diabetic female rats were coupled with low mRNA levels of PXR (CTRL: 1.23 ± 0.296; STZ: 0.686 ± 0.277, p=

0.002). In addition, as here observed, the NOR index, which is a measure of memory function, was significantly reduced in STZ female rats, and low PREG levels in female diabetic gut were positively correlated with this index. Thus, altogether, the results here obtained on PREG in this T1DM experimental model suggested not only a local role (i.e., in the gut) but also a role in the gut brain-axis (i.e., in controlling nervous function).

Overall, our study showed that memory alteration highlighted in STZ- induced female rats, is associated with different elements, both from CNS and periphery. In the CNS, it seems to be due to increased neuroinflammation (i.e., increase in the levels of IL-6 and IL-1β) and aberrant synaptogenesis (i.e., decrease in the levels of synaptophysin, synapsin and syntaxin) in hippocampus of STZ-female rats but there is also an attempt to response positively due to increased ALLO levels. Thus, it is evident that in the CNS neuroactive steroids have a pivotal role. Their involvement is also seen in the colon where PREG and ALLO levels are altered. Particularly, while the decreased levels of PREG correlate with behavioural results, supporting the cognitive impairment observed, the inflammatory pattern may have a role in the development of dysbiosis which in turn, by altering factors such as fatty acid levels or other signal molecules, may contribute to the alterations observed in the CNS.

In conclusion, the neuroactive steroids and gut steroids proved to be important molecules involved in female cognitive function observed in this T1DM experimental model. Further studies are necessary to understand the relationship between the pool of steroids produced in the CNS and those produced in the colon and their relevance for brain function. As a future perspective, the increased knowledge in the mechanisms linked to steroids and cognition will open new therapeutic strategies, also considering the context of gender medicine, to treat diabetic encephalopathy.

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