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PH.D. THESIS

MOLECULAR MECHANISMS OF DIABETES-ASSOCIATED DOPAMINERGIC DYSFUNCTION IN NEURONAL CELLS

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Ai miei genitori…

per tutto quello che hanno fatto
e che continuano a fare, in punta di piedi, per me,
anche quando non lo merito,

è anche grazie a voi se sono arrivata fino a qui…

A Maia e Giuseppe…

spero seguiate sempre il vostro istinto ed il vostro cuore
e che non vi fermiate mai di fronte alle difficoltà
che incontrerete lungo il cammino…

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Fiory F, Mirra P, Nigro C, **Pignalosa FC**, Zatterale F, Ulianich L, Prevete N, Formisano P, Beguinot F, Miele C. *Role of the HIF-1α/Nur77 axis in the regulation of the tyrosine hydroxylase expression by insulin in PC12 cells*. J Cell Physiol. 2019 Jul;234(7):11861-11870. doi: 10.1002/jcp.27898.

Fiory F, Perruolo G, Cimmino I, Cabaro S, **Pignalosa FC**, Miele C, Beguinot F, Formisano P, Oriente F. *The Relevance of Insulin Action in the Dopaminergic System*. Front Neurosci. 2019 Aug 16;13:868. doi: 10.3389/fnins.2019.00868.

Pignalosa FC, Desiderio A, Mirra P, Nigro C, Perruolo G, Ulianich L, Formisano P, Beguinot F, Miele C, Napoli R, Fiory F. *Diabetes and Cognitive Impairment: A Role for Glucotoxicity and Dopaminergic Dysfunction*. Int J Mol Sci. 2021 Nov 16;22(22):12366. doi: 10.3390/ijms222212366.

ABSTRACT

DA is a catecholamine that regulates several relevant body functions, including cognition. Interestingly, dopaminergic dysfunction and cognitive decline are both associated to diabetes mellitus, an eterogenic disorder characterized by alterations in glucose, lipid and protein metabolism. An interplay between glucose metabolism and dopaminergic function has been evidenced and it has been shown that relative insulin deficiency and chronic hyperglycemia, the two major players of diabetes, can affect the dopaminergic system, altering DA content and turnover. In particular, pathological states characterized by impaired insulin signaling are associated with alterations in expression and/or activity of TH, the rate limiting enzyme for DA synthesis, suggesting that insulin is able to modulate TH expression and thus DA synthesis. On the other hand, the hyperglycemia typical of DM leads to the intracellular accumulation of MGO, a reactive dicarbonyl, able to induce the non-enzymatic glycosylation of proteins and lipids and nucleic acids, contributing to glucotoxicity. MGO plays a key role in cognitive decline and in dopaminergic dysfunction. In particular, repeated intraperitoneal injection of MGO in mice reduces DA content in the prefrontal cortex and in neuronal cells MGO modulates the expression of genes involved in the metabolism of DA. However, the molecular mechanisms underlying insulin regulation of TH and MGO modulation of dopaminergic genes are still unknown. This work aims: 1) to clarify the molecular basis of insulin action on TH expression, identifying the transcription factors involved 2) to investigate MGO ability to induce dopaminergic dysfunction and to unveil the molecular mechanisms of MGO regulation of dopaminergic genes. Since in endothelial cells MGO regulates gene expression modulating levels of specific miRNA, a particular effort has been focused on the investigation of miRNA role in MGO modulation of dopaminergic genes. The results obtained show that: 1) Insulin modulates TH mRNA and protein levels in a biphasic manner in PC12 cells. The activation of insulin receptor and of insulin effectors PI3K and ERK1/2 are required for insulin-induced increase of TH expression. The transcription factors involved downstream insulin action are HIF-1alpha and Nur77. 2) MGO induces dopaminergic dysfunction reducing dopamine levels in SHSY5Y cells. This reduction is paralleled by an increased expression of genes involved in DA degradation (MAO A and COMT) and of SNCA gene, codifying for alpha synuclein, a protein involved in neurodegeneration and known to modulate DA amount. miR-214 and miR-190a are involved in MGO induced dopaminergic dysfunction and mediate MGO effect on the expression of COMT and SNCA.

In conclusion, the results showed in my PhD thesis shed light on the molecular mechanisms underlying diabetes associated dopaminergic dysfunction and represent the first step for the identification of innovative pharmacological targets potentially useful for treatment of diabetes associated dopaminergic dysfunction and cognitive decline.

1. BACKGROUND

1.1 Dopamine: metabolism and signaling

Neurotransmitters are chemical messengers synthesized in the central nervous system (CNS) and stored in presynaptic vesicles. They are released in the synaptic cleft following an action potential and induce a physiological response in postsynaptic or nearby cells, regulating cell-to-cell communication in the CNS (*Kanner & Schuldiner, 1987*). The removal of neurotransmitters from the synaptic cleft is very fast. It occurs in three different ways: 1) enzyme degradation, 2) reuptake by presynaptic neurons, 3) binding to specific receptors localized on postsynaptic or target cells for internalization. Neurotransmitters can be divided according to their molecular identity in small organic molecules, peptides, monoamines, nucleotides and amino acids, or according to their excitatory or inhibitory function (*Kandel et al. 2013*). The monoamine transmitters are small charged molecules unable to cross the blood-brain barrier, synthesized from amino acids by one rate-limiting enzymatic reaction (*Kortekaas et al. 2005*). Catecholamines are monoamines deriving from the amino acid tyrosine and include three essential neurotransmitters: norepinephrine, epinephrine and dopamine (DA, a contraction of 3,4-dihydroxyphenethylamine) (*Fernstrom and Fernstrom, 2007*). DA is a compound with positive ionotropic activity, belonging to phenethylamine families, that constitutes about 80% of the catecholamine content in the brain and plays a key role in brain and body function.

Chemically, DA consists of a catechol ring with one ethylamine group (Figure 1), and acts like an organic base which turns into protonated form in an acidic environment such as other amines. DA is soluble in water when in protonated form and is degraded when exposed to oxygen or other oxidants. In contrast, its basic form is highly reactive and less soluble in water (*Kaur et al. 2020*).

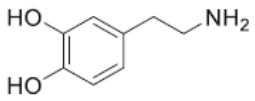

Figure 1: Dopamine structure.

The classical pathway of DA biosynthesis involves two steps. The first step is the hydroxylation of L-tyrosine to dihydroxyphenylalanine (L-DOPA), catalyzed by the rate-limiting enzyme tyrosine hydroxylase (TH) in the presence of tetrahydrobiopterin (BH_4),

O_2, and Fe^{2+} as cofactors (*Juárez Olguín et al. 2016*). Subsequently, L-DOPA is decarboxylated to the final product DA by aromatic L-amino acid decarboxylase (DOPA decarboxylase, DDC) in the presence of pyridoxal phosphate (PP) as a cofactor (*Meiser et al. 2013*) (Figure 2).

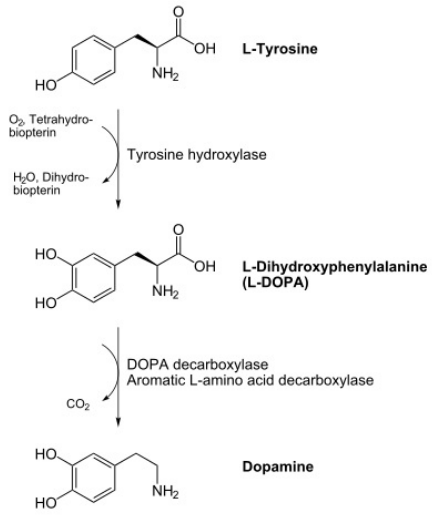

Figure 2: Classical DA biosynthesis pathway.

In addition to this classical biosynthetic pathway, there are two other pathways of DA synthesis: the *Cytochrome P450-mediated pathway*, where tyrosine is decarboxylated to tyramine and later hydroxylated by Cyp2D (Cytochrome P450 2D6) proteins; *Tyrosinase mediated pathway*, where tyrosine is hydroxylated by tyrosinase during melanin synthesis. Both these pathways contribute to DA synthesis in a lesser extent (*Meiser et al. 2013*).

Once synthesized, DA is readily sequestred into the synaptic vesicles through vesicular monoamine transporter 2 (VMAT-2) under acidic pH to prevents its oxidation, and remains stored until excitation of dopaminergic neurons. Upon stimuli, synaptic vesicles degranulate, releasing DA in the synaptic cleft, and excess of DA is removed by the dopamine transporter DAT or by plasma membrane monoamine transporter (*Kaur et al. 2020*).

To exert its action, DA released in the synaptic cleft binds to specific receptors located on the plasma membrane of target cells. DA receptors belong to the superfamily of G protein-coupled receptors (GPCRs) (*Klein et al. 2019*). These are metabotropic receptors and modulate levels of second messengers, which activate or repress specific cell signaling pathways (*Baik, 2013; Beaulieu et al. 2015*). DA receptors include five different subtypes:

D1, D2, D3, D4, and D5 and are divided in two groups according to their structural and pharmacological features and to their localization:

- **D1-like receptors**: including D1 and D5, localized only in postsynaptic DA-responsive cells. They are coupled to the stimulatory α subunit of G proteins (Gαs/olf) and increase adenylyl cyclase (AC) activation and cAMP production as a second messenger. This pathway, in turn, induces protein kinase A (PKA) with several downstream substrates and the immediate-early gene expression;
- **D2-like receptors**: including D2, D3, and D4, located both pre- and postsynaptically. They are coupled to the inhibitory α subunit of G proteins (Gαi) that negatively regulate cAMP production, inhibiting adenylyl cyclase and PKA (*Baik, 2013; Beaulieu and Gainetdinov, 2011*).

As well as regulating AC activity, DA receptors can also couple to the stimulatory αq subunit of G proteins, modulating phospholipase C (PLC) activity. PLC catalyzes the production of inositol trisphosphate and diacylglycerol, which respectively increase intracellular calcium levels and activate protein kinase C (PKC) (*Klein et al. 2019*) and Ca^{2+}/calmodulin-dependent protein kinase (CAMKII) (*Kebabian,1978; Cantrell et al. 1997*). The PKC pathway activation in turns leads to a potentiation of PKA signaling. β-arrestin 2 is involved in DA receptors signaling and regulation, too. Indeed, it binds to phosphorylated D2 receptors and leads to the formation of a complex that includes the serine-threonine kinase AKT and the phosphatase PP2A, resulting in constitutive activation of two AKT substrates, GSK3α and GSK3β (*Felder et al. 1989*). Furthermore, β-arrestin 2 binding to DA receptors induces their downregulation through the internalization by a clathrin-mediated pathway (*Beaulieu et al. 2005*) (Figure 3).

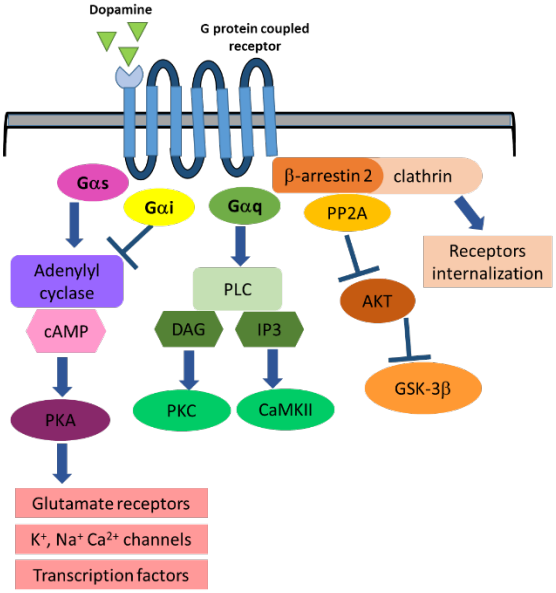

Figure 3: Main pathways involved in DA signaling (*Pignalosa et al. 2021*).

In the synapse, DA binds presynaptic receptors, postsynaptic receptors or both. When DA binds presynaptic receptors, also known as autoreceptors with an inhibitory potential, its further synthesis and its release are inhibited to keep physiological levels of DA. In the case of activation of postsynaptic DA receptors, the signal is propagated to the postsynaptic neurons (*Juárez Olguín et al. 2016*).

Once exerted its function by interacting with DA receptors, DA is reuptaken in the cytosol of presynaptic cells through high-affinity DA transporters (DAT) or low-affinity plasma membrane monoamine transporters (*Juárez Olguín et al. 2016*). In the cytosol, DA can be repackaged into vesicles by VMAT-2, being available for future release, or can be broken down by monoamine oxidase (MAO) or catechol-O methyltransferase (COMT) (*Kaur et al. 2020*). MAO exists in two isoforms, MAO-A and MAO-B. MAO-B is found in astrocytes, while MAO-A is found predominantly in catecholaminergic neurons of the SN. MAO degrades DA in 3,4-dihydroxyphenylacetaldehyde (DOPAL) which is then degraded by aldehyde dehydrogenase in 3,4-dihydroxyphenylacetic acid (DOPAC) (*López-Pérez et al. 2015*). At variance, the enzyme COMT converts DA to 3-methoxytyramine (3-MT). Then, 3-MT is reduced to homovanillic acid (HVA) by the action of MAO and eliminated in the urine (*Juárez Olguín et al. 2016*).

It is worthy of notice that extracellular DA levels are finely tuned through two main mechanisms: phasic and tonic transmission (*Floresco et al. 2003*). Action potentials in the

DA-containing cells drive phasic DA release, resulting in a rapid and transient increase of DA concentrations in the synaptic cleft due to synchronized burst firing. DA release during phasic transmission determines an immediate saturation of the D2 receptor. At the same time, the activity of DAT regulates DA extacellular levels through reuptake, and the concentrations of the neurotransmitter vary in the order of milliseconds. On the other hand, tonic transmission occurs when DA is released without presynaptic action potentials, and it is regulated by the activity of other neurons and neurotransmitter reuptake or degradation (*Floresco et al. 2003*). Tonic release generates milder increases in extracellular DA, compared to phasic release (*Venton et al. 2003*), resulting in nanomolar concentrations of DA, ranging in scale from seconds to minutes. Even if the tonic DA concentration is lower than phasic transmission, it is sufficient to stimulate the presynaptic D2 receptors present on the afferent terminals (*Floresco et al. 2003*).

1.2 Anatomy of the dopaminergic system and dopamine functions

There are four main dopaminergic pathways in the human CNS. The ventral tegmental area (VTA) is the source of the mesocorticolimbic system, divided into the mesolimbic pathway and mesocortical pathway. Dopaminergic neurons project their axons from VTA towards the cortex via the mesocortical way and to the *nucleus accumbens* via the mesolimbic route (*Kelley & Berridge, 2002*). In the *substantia nigra*, dopaminergic neurons project their extensions towards the striatum and form the nigrostriatal pathway (*Hikosaka et al. 2002*). The tuberoinfundibular pathway is formed when the axons of dopaminergic neurons go from the hypothalamic nuclei (arcuate nucleus and paraventricular nucleus) towards the pituitary gland (*Klein et al. 2019*) (Figure 4). These pathways are involved in different function: mesocortical and mesolimbic pathways are involved in reward and motivation; the nigrostriatal pathway is involved in motor function and learning ability (*Klein et al. 2019*), and in pain modulation (*Magnusson & Fisher, 2000*); the tuberoinfundibular pathway plays an important role in regulating the secretion of prolactin by the pituitary gland (*Demarest et al. 1984*).

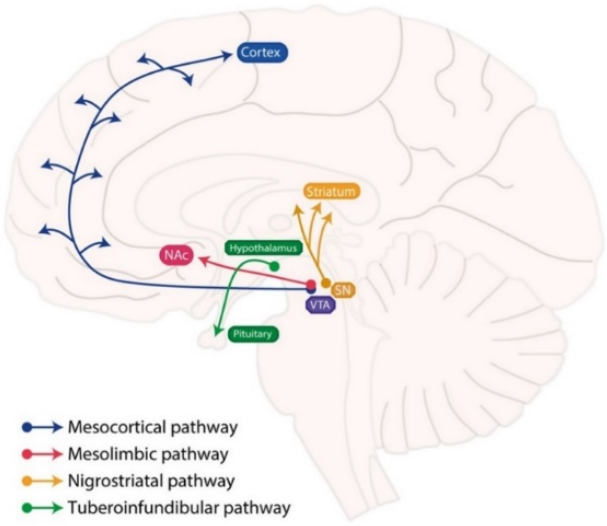

Figure 4: Dopaminergic pathways in the CNS (*Klein et al. 2019*).

More in detail, DA controls many different body functions. Indeed, the dopaminergic system plays important roles in movement, motor control, cognitive function (*Paus, 2001*), spatial memory function (*Luciana et al. 1998*), arousal (*Andretic et al. 2005*), reinforcement, reward, affect (*Berridge & Kringelbach, 2008*), sleep regulation (*Grossman et al. 2000*), motivation (*Depue & Collins, 1999*), attention (*Aston-Jones, 2005*), feeding, olfaction, and hormone regulation (*Li C et al. 1999*). Moreover, DA influences also the immune (*Basu & Dasgupta, 2000*), cardiovascular (*Contreras et al. 2002*), gastrointestinal (*Willems et al. 1985*) and renal systems (*Aperia, 2000*). Even in the retina, the extracellular release of DA during daylight enhances the activity of cone cells and increase sensitivity to color and contrast (*Hampson et al. 1992*). Lastly, DA is involved in nausea (*Nakagawa et al. 2008*), lactation (*Demarest et al. 1984*), maternal and reproductive behaviors (*Kruger et al. 2005*). However, two of the most relevant DA roles are the regulation of motor function and cognitive function.

1.2.1 Dopamine and motor function

DA acts as a critical interface between sensory function and voluntary movements, allowing the selection of motor plans appropriate to specific environmental and behavioral contexts (*Kosobud et al. 1994; Mirenowicz & Schultz, 1996; Schultz, 2010*). From an anatomical point of view, the *substantia nigra pars compacta*, together with the VTA, is responsible for the regulation of motor function through the basal ganglia (*Pérez-*

Fernández et al. 2021). Dopaminergic system is deeply involved in this regulation, as evidenced by motor deficits that appear in Parkinson's disease (PD) when ascending dopaminergic projections from the *substantia nigra pars compacta* to the basal ganglia are compromised (*Pérez-Fernández et al. 2021*). In turn, the basal ganglia project to a brainstem region, modulating locomotion, called MLR (Mesencephalic Locomotor Region) (*Ryczko & Dubuc, 2013*). Moreover, a descending dopaminergic pathway innervating MLR from *substantia nigra pars compacta* has been recently found (*Ryczko & Dubuc, 2017*). The ascending dopaminergic projections mostly target the striatum, where neurons expressing D1 or D2 receptors form separate populations (*Roseberry et al. 2016; Freeze et al. 2013*). Here, DA increases the excitability of D1-expressing neurons, while decreasing that of D2-expressing neurons (*Ericsson et al. 2013*), reducing the tonic inhibition exerted by basal ganglia to the MLR and inducing locomotion initiation.

1.2.2 Dopamine and cognitive function

Since 1965, the undeniable association between striatal DA depletion and motor deficits characteristic of PD is known (*Bernheimer & Hornykiewicz, 1965*). Later, it was shown that PD is characterized also by alterations of several cognitive domains, including executive functions (*Dalrymple-Alford et al. 1994*), attention (*Downes et al. 1989*), verbal fluency, visuospatial skills (*Boller et al. 1984*), episodic memory (*Warburton, 1967*), and reasoning (*Wilson et al. 1980*). Then, imaging studies performed in PD patients revealed a positive correlation between decreased DA levels and cognitive impairment (*Brück et al. 2001; Sawamoto et al. 2008; Jokinen et al. 2009*). Interestingly, also aging-associated cognitive decline is accompanied by various modifications of the dopaminergic system (*Lebowitz & Khoshbouei, 2020*), such as reduction of DA transporters and receptors density. In detail, in healthy subjects, molecular imaging studies highlighted an age-related decrease in striatal DA transporter (DAT) density, paralleled by a worse performance on several tasks, including episodic memory, executive functioning, and verbal learning tasks (*Erixon-Lindroth et al. 2005*). Similarly, there is also a loss of D2 receptors in both striatal and extrastriatal areas and simultaneous cognitive deficits, in healthy aging (*Bäckman et al. 2000*). In parallel, an increase of DA catabolism (*Fowler et al. 1980*) and alterations of DA synthesis were observed (*Berry et al. 2016; Juarez et al. 2019*). Thus, it is likely that dopaminergic dysfunction could contribute to cognitive decline. To confirm this, an abnormal dopaminergic function was found in several diseases characterized by cognitive impairment, such as schizophrenia (*Swerdlow & Koob, 1987*), attention deficit

hyperactivity disorder (ADHD) (*Del Campo et al. 2011*), Alzheimer's disease (*Nobili et al. 2017*), addiction (*Nutt et al. 2015*) and Huntington's chorea (*Schwab et al. 2015*). Taken together, these results strongly suggest the idea that DA plays a key role in cognitive function, too (*Guo et al. 2018; Martinussen et al. 2005; Aarsland et al.* 2017) and several *in vivo* data link DA release to cognitive function. For example, *in vivo* microdialysis experiments revealed increased DA release in the prefrontal cortex (PFC) of rats (*Phillips et al. 2004*) and monkeys (*Watanabe et al. 1997*) during working memory tasks, resulting in modulation of neuronal activity of the "memory field" of PFC neurons (*Yang & Seamans, 1996*). The importance of DA in cognitive function was definitely confirmed by experimental manipulation of the dopaminergic system in animal models and by pharmacological studies in humans. Studies performed in rodents aimed to clarify the involvement of DA in cognitive function (*Simon et al. 1986*). Simon and coworkers showed that, in rats, the bilateral injection of 6-hydroxydopamine (6-OHDA) into the lateral septum selectively abolishes dopaminergic innervation and leads to deficits in spatial-memory tasks, without significantly damaging endogenous noradrenergic and cholinergic systems (*Simon et al. 1986*). Similarly, in rhesus monkeys, depletion of DA in PFC seriously impairs working memory (*Brozoski et al. 1979*). The entity of the deficit is comparable to the one observed when ablation of the PFC itself occurs. Interestingly, there are no alterations in working memory in monkeys subjected to depletion of other neurotransmitters (*Cools et al. 2019*). This conclusion was further confirmed by the results obtained in rhesus monkeys where locally injection of selective DA D1 receptor antagonists into the prefrontal cortex lead to alteration of mnemonic processes (*Sawaguchi & Goldman-Rakic, 1991*). Experiments with D1 receptor agonists and antagonists strenghtened the idea that cognitive function is highly dependent on DA levels. Indeed, both excessive and inadequate activation of the D1 receptor alters the so-called working memory, both in monkeys and rodents (*Zahrt et al. 1997*). In parallel, human studies revealed that treating healthy subjects with bromocriptine, a D2 receptor agonist, improves spatial working memory (*Luciana et al. 1992*). Similarly, the administration of pergolide, an agonist for both D1 and D2 family receptors, improved working memory tasks' performance (*Kimberg & D'Esposito, 2003*). More detailed dose-response experiments with dopaminergic drugs support the hypothesis that the complex functional relationship between DA and working memory is governed by an inverted U-shaped, non-linear dose-response curve. Thus, both low and excessive doses of DA affect memory performance (*Cools & D'Esposito, 2011*). This trend is likely influenced by baseline DA levels and may

be due to the differential activation of DA receptors in the striatum and PFC. More recently, the effects of D1 and D2 receptor agonists have been further investigated by exploring different cognitive domains, such as memory, flexibility, and learning in non-human Primates (*Marino & Levy, 2019*). Indeed, cognitive impairment is often due not only to PFC malfunction but also to reduced transmission of striatal DA. Studies with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in animal and human models revealed the relevance of dopaminergic signaling in the striatum for cognitive function. Stern Y and colleagues in 1990 studied general intellectual function, construction, language, memory, executive function, attention, and reaction time in individuals exposed to MPTP, characterized by reduced absorption of 6-fluorodopa in the striatum. These subjects performed worse in a specific set of cognitive functions mediated by the dopaminergic system (*Stern et al. 1990*). Similarly, intranigral administration of MPTP in rats causes a partial lesion in the *substantia nigra pars compacta*. This results in a loss of DA in the striatum, inducing a deficit in learning and working memory (*Braga et al. 2005*). Several experiments support the role of DA also in the regulation of attention. Indeed, in rats, lesions of the terminal area of the nigrostriatal system induce partial denervation of the striatum, leading to attention deficit (*Amalric et al. 1995*), and in patients with ADHD, the stimulant drug methylphenidate improves attention by enhancing DA signaling in the striatum (*Volkow et al. 2012*).

1.3 Dopaminergic system and its alterations

Considering the wide range of effects downstream the activation of dopaminergic receptors, it is not surprising that dopaminergic dysfunction is implicated in the pathogenesis and progression of several diseases (*Klein et al. 2019*).

- *Parkinson's Disease*

Parkinson's disease (PD) is the most common neurodegenerative disorder caused by the loss of dopaminergic neurons in the *substantia nigra pars compacta* and by the presence of intraneuronal aggregates called Lewy Bodies enriched in a particular protein, α-synuclein (SNCA) (*Spillantini et al. 1997; Dexter & Jenner, 2013*). PD mostly affects the population over the age of 60 and has an incidence of about 150-200 cases per 100,000 individuals, with a higher prevalence in the male than in the female population. It is characterized by a set of parkinsonism motor symptoms, such as bradykinesia, resting tremor, rigidity, and postural instability. These symptoms, as well as non-motor symptoms

such as depression and cognitive impairment, are commonly attributed to striatal DA loss (*Dexter & Jenner, 2013*). PD was once considered as a sporadic disease, but the contribution of both common and rare genetic mutations has now been recognized. Indeed, genome-wide association studies have evidenced that common variations in 41 different loci modulate the risk of developing PD (*Chang et al. 2017*). Similarly, rare mutations have been found in familial forms of PD in leucine-rich repeat kinase 2 (LRKK2), SNCA, PTEN-induced putative kinase 1 (PINK1), parkin (PRKN), and DJ-1 genes (*Hernandez et al. 2016*). Many of these genes are involved in autophagy, lysosomal function, and mitochondrial integrity. These processes may contribute to the loss of dopaminergic neurons in PD (*Hauser & Hastings, 2013; Beilina and Cookson, 2016*). In the *substantia nigra pars compacta*, dopaminergic neurons are reduced by about 4% per decade during the normal aging process (*Fearnley & Lees, 1991*). However, this process is accelerated in PD, and parkinsonisms appear when the number of dopaminergic neurons and striatal dopaminergic terminals is reduced by 70% and 40%, respectively, compared to that expected in age-matched control subjects (*Cheng et al. 2010*).

- *Huntington's Disease*

Huntington's disease (HD) is an autosomal dominant disease caused by an expansion of the CAG triplet on exon 1 of the Huntingtin gene (HTT) (*Kim & Fung, 2014*). It is a progressive and fatal neurodegenerative disease that affects about 5 to 7 people per 100,000 individuals. The average age of onset is around 40 years, although, in some patients, this disease is diagnosed later in life. HD symptoms include movement dysfunction, such as chorea, but cognitive deficits and psychiatric disorders often appear as the disease progresses (*Kim & Fung, 2014; Cepeda et al. 2014*). The neurodegeneration caused by HD occurs in two portions of the brain that contain high levels of dopaminergic innervation and receptors. Therefore, DA signaling dysfunction is crucial to the symptoms of HD. Several studies have shown a significant reduction in DA binding to striatal D1 and D2 receptors in the brains of HD patients compared to healthy subjects (*Beaulieu & Gainetdnov, 2011*). Activation of D1 and D2 receptors leads to different consequences in HD. The early stages of HD are, indeed, characterized by hyperkinetic movements caused by an increase in thalamocortical glutamatergic signaling. Several studies have shown that the DA and glutamate pathways have a synergistic action that can increase toxicity through D1 receptor activation, promoting cell death. Indeed, it increases NMDA (Glutamate N-methyl-D-aspartate) responses that can promote neuronal death, while the activation of the

D2 receptor plays a protective role, decreasing NMDA receptor response (*Cepeda et al. 2014*).

- *Schizophrenia*

Schizophrenia (SZ) is a severe mental illness that presents a heterogeneous combination of symptoms. Three types of symptoms characterize it: positive (hallucinations, disorganized speech), negative (impaired motivation and social isolation), and cognitive (memory dysfunction). SZ affects 1% of the world population, and it is more frequent and more severe in men than women. The SZ onset occurs in late adolescence and is uncommon after age 50 (*Owen et al. 2016; Meyer & MacCabe, 2016*).

Although the neurobiology of SZ needs elucidation, several hypotheses have been made, strongly suggesting the involvement of DA (*Howes et al. 2017*). Clear evidence supports the idea that impaired dopaminergic transmission and abnormalities in glutamate signaling are involved in the genesis of psychotic and cognitive symptoms (*Owen et al. 2016*). *In vivo* studies on cognitive function show that patients with schizophrenia feature a high density of D2 receptors without alterations in the levels of D1 receptors, compared to healthy subjects. (*Beaulieu and Gainetdinov, 2011*). Changes in neuronal circuit function can be regulated by the homologous and heterologous interaction of D1 and D2 receptors, as well as by other signaling proteins (*Xu et al. 2017*).

- *Attention Deficit/Hyperactivity Disorder*

Attention/deficit Hyperactivity disorder (ADHD) is a behavioral and multifactorial psychiatric disease prevalent in childhood with a complex etiology and strong genetic basis (*Matthews et al. 2014*). About 5% of children and about 2.5% of adults have ADHD (*Nigg, 2013*). In about 80% of children diagnosed with ADHD, symptoms persist into adulthood (*Faraone et al. 2003*). ADHD is characterized by inattention (daydreaming and distraction), impulsivity, excessive motor activity, and hyperactivity (*Lange et al. 2010; Matthews et al. 2014*). In animal models of ADHD, motor hyperactivity is associated with dopaminergic dysfunction. Functional studies in humans confirmed the role of dopaminergic dysfunction in the neurobiology of ADHD (*Castellanos & Tannock, 2002; Viggiano et al. 2002*). Faraone and co-workers showed that, in human, specific genes are associated with ADHD. These genes are related to the catecholamine signaling system components and include DAT, the norepinephrine transporter (NET), D4 and D5 receptors (*Faraone et al. 2005*). Accordingly, DAT knock-out mice exhibit hyperactivity and deficits

in inhibitory behavior, two features of ADHD. Abnormal levels of DAT have also been found in the brains of ADHD patients and DAT is a target of anti-hyperactivity drugs. In addition, meta-analysis studies show the presence of childhood ADHD-associated polymorphisms in genes encoding D4 and D5 receptors (*Faraone et al. 2005*).

- *Addiction*

Addiction is one of the most significant social and public health problems, and after obesity, it is the leading cause of preventable death in the United States associated with the use of substances such as tobacco and alcohol (*Lynch et al. 2013; Nutt et al. 2015*). Addictions are chronic relapsing disorders characterized by three essential components: compulsive drug use, the inability to limit drug intake, and the onset of a withdrawal syndrome with the presence of anxiety, irritability, and dysphoria (*Koob et al. 1998*). Even though there have been more than 40 years of scientific studies since the discovery of DA's involvement, the neurobiology of addiction remains to be elucidated in order to promote the development of therapeutic strategies (*Nutt et al. 2015*). Strong evidence indicates that synaptic alterations in the mesolimbic system are linked to food and drug addiction. Drug addiction patients show a low level of D2 receptors in the striatal area, and PET studies suggest less availability of these receptors (*Baik et al. 2013*). Studies conducted in rats chronically exposed to drugs, such as cocaine, showed an adaptation of the dopaminergic system in the ventral tegmental area caused by an upregulation of the D1 receptors, and a downregulation of the D2 receptors. These data indicate the possible role played by DA in the regulation of addiction, although its neurobiology needs to be further elucidated (*Lynch et al. 2013*).

1.4 Dopaminergic dysfunction and cognitive decline in diabetes

Diabetes mellitus (DM) is a heterogeneous metabolic disorder characterized by chronic hyperglycemia, representing a global epidemic public health problem (*ADA, 2019*). Type 1 and type 2 diabetes, the two most common forms of DM, differ in epidemiology and etiology. Type 1 diabetes (T1D) is primarily a juvenile-onset disease due to autoimmune destruction of pancreatic β-cells, leading to an absolute deficiency in insulin production. Type 2 diabetes (T2D) includes 90–95% of diabetes cases and is a pathology typical of the elderly, resulting from insulin resistance accompanied by progressive β-cells deficit (*Petersmann et al. 2019*). Premature morbidity and mortality of DM are due to long-term diabetic complications (*Harding et al. 2019*), including retinopathy, nephropathy,

peripheral vascular disease, and heart disease (micro- and macro-vascular disease), caused by deleterious action of hyperglycaemia in several anatomical areas (*Reasner, 2008*).

Several experimental data highlighted an interplay between glucose metabolism and the dopaminergic system. Indeed, it has been shown that modulation of striatal and systemic DA levels impinges on whole-body glucose metabolism (*Ter Horst et al. 2018*) and energy homeostasis (*Wang et al. 2001; de Weijer et al. 2011*). Interestingly, activation of D1 and D2 receptors affects insulin sensitivity (*Lacau-Mengido et al. 2000; Ter Horst et al. 2018*) and modulates insulin secretion (*García-Tornadú et al. 2010*) respectively, improving glucose tolerance (*Díaz-Torga et al. 2001*), both in rodents and in humans. Accordingly, systemic DA depletion leads instead to a reduction of insulin sensitivity in healthy subjects (*Ter Horst et al. 2018*), as well the pharmacological inhibition of DA receptors induces hyperinsulinemia and glucose intolerance (*Tarricone et al. 2006; Ballon et al. 2014*). On the other hand, alterations of dopaminergic system have been often observed in DM. Indeed, DM promotes neurodegeneration and impairs dopaminergic neurotransmission (*Pérez-Taboada et al. 2020*). Some authors described increased levels of DA during DM in specific brain regions of alloxan- or streptozotocin (STZ) rats (*Ramakrishnan et al. 2005; Lacković & Salković, 1990*), as well as in diabetic patients (*Lacković et al. 1990*). The selectivity of the alterations in the DA content was further confirmed by studies in which DA amount is reduced in the cerebral cortex, midbrain, and brain stem regions but increased in the cerebellum and thalamus/hypothalamus (*Ezzeldin et al. 2014*). In recent years, more detailed studies supported the hypothesis of a decrease in DA levels in different areas of the brain in DM. In particular, a reduction in DA levels was observed in the hippocampus of STZ and WBN/Kob rats. Further studies show that the selective reduction of DA content in the nigrostriatal system of STZ rats and the alteration of catecholamine metabolism depends on the severity and duration of DM (*Gallego et al. 2003*). Similar results were obtained in studies showing low DA levels and low related metabolites both in the striatum of STZ-treated mice and in db/db diabetic mice. Furthermore, these studies also evidenced a reduction in the expression of proteins that regulate DA's neurotransmission and stimulus-dependent striatal DA release, such as DAT, VMAT2, and Girk2 (G protein-activated inward rectifier potassium channel 2) (*Pérez-Taboada et al. 2020*). It is worthy of notice that in DM, the expression of some proteins involved in the synthesis and degradation of DA, such as TH, MAO, COMT, SNCA, is altered. The harmful effects of DM on TH function are known since 1980s, when alterations in the

content of DA precursor amino acids and changes in TH activity were observed in STZ rats (*Kwok & Juorio, 1986*). In addition, TH mRNA levels were increased in the locus coeruleus and decreased in the VTA/*substantia nigra pars compacta* of STZ-treated rats (*Figlewicz et al. 1996*). The deleterious effect of DM on the dopaminergic system has recently also been confirmed by human studies. In fact, in the cerebrospinal fluid of diabetic patients, there are deficiencies in the striatal dopaminergic system and high levels of proteins involved in neurodegeneration, such as tau and SNCA (*Pagano et al. 2018*).

Interestingly, the reduced DA content in the hippocampus of STZ rats goes hand in hand with the upregulation of DRD1 and DRD2 expression and contributes to the onset of cognitive deficit (*Yamato et al. 2004; Robinson et al. 2009*). Accordingly, since 1922 (*Miles & Root, 1922*), emerged the idea that a worsening of cognitive function accompanies both T1D (*Bispham et al. 2020*) and T2D (*Perlmuter et al. 1984*). Epidemiological studies definitely showed that DM is associated with an increased risk of dementia (*Biessels et al. 2006*) and less serious cognitive dysfunctions (*Koekkoek et al. 2015*). The intensity of cognitive deficit depends on diabetes type, age of onset, co-occurrence of complications, and comorbidities (*Biessels et al. 2008*). Nowadays, cognitive decline can be considered a well-established complication of DM (*Nelson et al. 2009*). Different factors are involved in its pathogenesis, including diabetic macro- and micro-angiopathy, cerebral vascular injury, amyloid and tau accumulation, poor glycemic control and neurodegeneration, due to oxidative insult and mitochondrial dysfunction (*Liu Y et al. 2011*). However, among DM complications, the cognitive deficit remains the less addressed and the research in this field is still ongoing. Given the key role of DA transmission in cognitive function, the hypothesis that alterations of the dopaminergic system observed in DM could be involved in the pathogenesis of DM associated cognitive decline is intriguing. To date, the interplay between DA function and glucose metabolism is well assessed and it is clear that the two major players of DM, hyperglycemia and relative insulin deficiency, can alter the dopaminergic system.

1.4.1 Impact of insulin resistance on dopaminergic system

Insulin is a peptide hormone secreted in response to postprandial hyperglycemia by pancreatic β-cells. Historically, insulin is the principal regulator of peripheral glycemic homeostasis, as it stimulates glucose uptake by adipose tissue and skeletal muscle. Insulin also induces glycogen synthesis in the liver and, in parallel, inhibits hepatic

gluconeogenesis and glycogenolysis (*Haeusler et al.2018*). In addition to these peripheral targets, insulin also has a neuroregulatory function. It is known that insulin enters the brain parenchyma and precapillary spaces through receptor-mediated transport and that insulin transport to the CNS is reduced by hyperglycemia (*Banks et al. 1997*). Detectable insulin concentrations have been found in different brain regions, such as the hypothalamus, olfactory bulb, and midbrain. However, it is not clear if insulin is produced locally in the CNS (*Baskin et al. 1983*). Interestingly, Alzheimer's and aging are associated with lower insulin transport across the blood-brain barrier, suggesting a role for insulin in CNS (*Craft et al. 1998; Frölich et al. 1998*). However, the physiological significance of its action in the brain only recently was better clarified in mouse models and human studies (*Schubert et al. 2004; Grote & Wright; 2016*). Insulin elicits its effects by binding to a specific tyrosine kinase receptor expressed in different brain regions (*Plum et al. 2005*), including dopaminergic neurons (*Figlewicz et al. 2003; Könner et al. 2011*), and represents a well-known key regulator of both neuronal survival and DA metabolism. Indeed, it protects rat hippocampal cells in culture by oxygen-glucose deprivation (*Mielke & Wang, 2005*) and has neuroprotective action against H_2O_2 in retinoic acid (RA)-differentiated SH-SY5Y cells (*Ramalingam & Kim, 2014*). Similarly, insulin protects dopaminergic neurons of *substantia nigra pars compacta* against 6-OHDA toxicity, in rats (*Pang et al. 2016*). Moreover, in *ex vivo* differentiated human dopaminergic neurons and in SH-SY5Y cells culture, insulin resistance is accompanied by mitochondrial dysfunction, increased ROS levels, and increased expression of SNCA (*Hong et al. 2020*). Significantly, a compromised insulin signaling alters DA homeostasis and function (*Carvelli et al. 2002; Garcia et al. 2005*). Indeed, the ablation of insulin receptors in dopaminergic neurons interferes with DA control of food intake (*Könner et al.* 2011), and insulin is also able to regulate DA turnover, increasing DAT-mediated DA uptake (*Patterson et al. 1998; Speed et al. 2010*). Interestingly, brain-specific insulin receptor knock-out mice (NIRKO mice) are characterized by increased DA turnover in the striatum and *nucleus accumbens*, resulting in reduced DA signaling (*Kleinridders et al. 2015*). DA synthesis can be modulated by insulin, too. Evidence in the literature shows that pathological states characterized by an alteration of insulin signaling are associated with alterations in TH's expression and/or activity (*Glanville & Anderson, 1986; Li Y, South T et al. 2009; Morris et al. 2011; Nascimento et al. 2011*). For example, high-fat-fed animals exhibit insulin resistance and more significant DA depletion than chow-fed animals, both in the *substantia nigra* and in the striatum after 6-OHDA infusions, supporting the idea that there is a relationship

between insulin resistance and DA metabolism (*Morris et al. 2010*). Deficits in DA terminal function, observed in high fat-fed insulin-resistant mice, are reverted when insulin receptor signaling is restored (*Fordahl & Jones, 2017*). Interestingly, in 6-hydroxy-DA injected rats treated with intranasal insulin significantly improves motor impairments and increases TH positive neurons in the *substantia nigra pars compacta* (*Pang et al. 2016*). Accordingly, Ramalingam and Kim showed that insulin has a protective effect on MPP+-induced neurotoxicity in SHSY5Y cells, increasing TH levels (*Ramalingam & Kim, 2016; Perruolo et al. 2016*). Taken together, all this evidence suggests that insulin may play a key role in modulating TH expression and dopaminergic function, but the molecular mechanisms *underlying* this event are still unknown.

1.4.2 Impact of hyperglicemia on dopaminergic system

An adequate intake of glucose in the brain is essential for the homeostasis of dopaminergic neurons and DA metabolism. Studies focusing on the effect of hyperglycemia on dopaminergic neurons revealed that they are driven into apoptosis by chronic glucose exposure through oxidative damage (*Su et al. 2020*). In PC12 cells, chronic high glucose incubations increase depolarization-induced DA release and, in healthy subjects, blood glucose levels are correlated with homovanillic acid concentrations in cerebrospinal fluid (*Koshimura et al. 2003; Umhau et al. 2003*). In rats, variations in glucose levels in the *substantia nigra* produce different effects on DA release. The action of glucose also appears to involve the K^+ ATP-dependent channels and affects the release of other neurotransmitters. However, glucose infusions seem to increase DA release when glucose availability is low in the nigrostriatal pathway. On the contrary, it decreases DA release when glucose is abundant (*Levin, 2000*). Together with intracerebral glucose concentrations, systemic glucose levels can affect dopaminergic system, too. Very recently, dopaminergic changes induced by chronic hyperglycemia have been studied in STZ rats. The high vulnerability of the nigrostriatal dopaminergic system to chronic hyperglycemia results from high basal levels of oxidative stress coupled with low levels of antioxidant defenses (*Renaud et al. 2018*). Chronic hyperglycemia**,** typical of DM, seriously damages organs and tissues, causing DM complications through several mechanisms, including the increased activation of the polyol and hexosamine pathway, the protein kinase C (PKC), and the increased intracellular production of AGEs (*Brownlee, 2005*). Interestingly, hyperglycemia has a set of adverse effects better identified with the word “glucotoxicity”.

1.4.3 Glucotoxicity

Glucotoxicity is characterized by the abnormal accumulation within the cell of reactive carbonyls, such as methylglyoxal (MGO), glyoxal, and 3-deoxyglucosone (*Zheng et al. 2016*). MGO, represents the most potent alpha-ketoaldehyde among the glycating agents, with a reactivity between 10,000 and 50,000 times greater than glucose. The synthesis of MGO occurs through several metabolic pathways: 1) as a by-product of glycolysis, following the spontaneous degradation of glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP). 2) following the metabolism of ketone bodies (oxidation of acetoacetate and acetone). 3) from the catabolism of threonine; 4) from the fragmentation of Amadori's products and 5) from the peroxidation of lipids (*Bellier et al. 2019*) (figure 6).

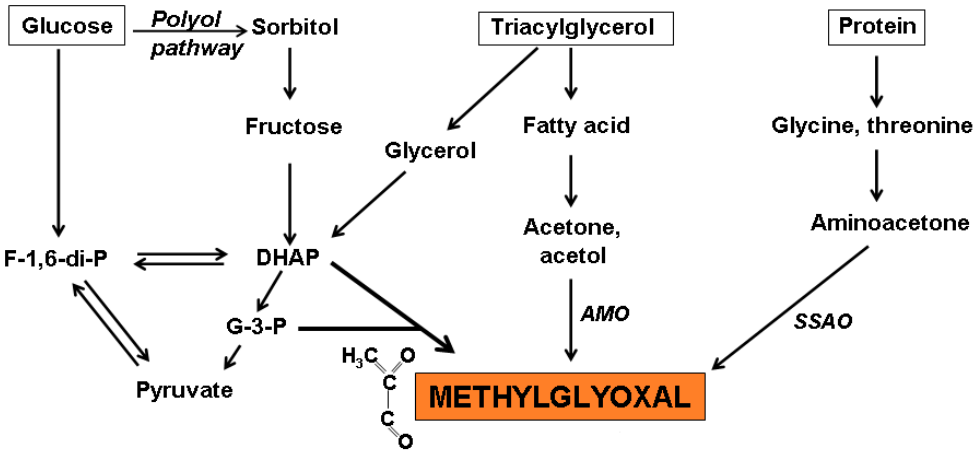

Figure 5: Biochemical pathways leading to MGO formation. F-1,6-di-P, fructose 1,6-bisphosphate; DHAP, triosephosphates dihydroxyacetone phosphate; G-3-P, glyceraldehyde-3-phosphate; SSAO, semicarbazide-sensitive amine oxidase; AMO, amine oxidase (*Desai et al. 2010*).

Given its reactivity, cells have an enzymatic system for detoxifying MGO. Glyoxalase 1 (GLO1) and glyoxalase 2 (GLO2) are key enzymes in the anti-glycant defense, catalyzing the conversion of MGO into D-lactate in the presence of GSH. The detoxification of α-oxoaldehydes, mediated by GLO1, plays a fundamental role in conditions of diabetes and uremia, where MGO concentrations are increased.

Minor detoxification systems include aldehyde dehydrogenase (ALDH) and aldose reductase (AR), induced when glyoxylase is inactive (*Hanssen et al. 2019*). The interaction of MGO with amino acid residues induces oxidative stress and free radical production. This effect can also be generated following glycosylation and inactivation of antioxidant

enzymes. MGO is able to react with proteins, lipids, and nucleic acids in a non-enzymatic glycation reaction. In particular, MGO reacts with arginine and lysine residues of proteins and deoxyguanosines of the DNA, giving rise to advanced glycation end products (AGEs) and to MGO-derived DNA adducts, respectively. Importantly, the interaction of MGO with amino acids and nucleic acids alters the structure and functionality of these molecules and induces cytotoxicity and apoptosis as a final effect. For istance, the high levels of MGO found in diabetes conditions cause apoptosis of pericytes of the bovine retina through the induction of oxidative stress and the activation of NF-kB (*Kim et al. 2004*).

Many studies on animal models and cultured cells highlighted MGO involvement in the pathogenesis and progression of diabetes and insulin resistance. Indeed, MGO is able to induce insulin resistance in adipose tissue (*Jia & Wu, 2007*), skeletal muscle (*Riboulet-Chavey et al. 2006*), endothelial cells and β-cells. Moreover, it damages also β-cell, decreasing glucose induced insulin secretion and the expression of genes essential for secretory function, such as as INS1 (insulin 1), GCK (glucokinase) and PDX1 (pancreatic and duodenal homeobox 1) (*Fiory et al. 2011*). The molecular mechanisms underlying MGO action on insulin sensitivity have been only partially elucidated. They include reduced efficiency of Glo1 detoxification (*Nigro et al. 2014*), impairment of GLUT-4 trafficking and the formation of AGEs adducts on IRSs. Interestingly, in endothelial cells, MGO alters insulin-induced activation of the PI3K/Akt/eNOS (endothelial nitric oxide synthetase) signaling pathway, increasing the phosphorylation of ERK1/2 (extracellular signal-regulated protein kinase 1/2), and determining an imbalance in the release of two important vasoactive molecules of the endothelium: endothelin1 (vasoconstrictor) and nitric oxide (NO) (vasodilator) (*Nigro et al. 2014*). Recent studies show that MGO can also modulate gene expression by modifying the levels of endogenous small molecules of non-coding RNA (miRNA) (*Mirra et al. 2017; Nigro et al. 2018*). Numerous miRNAs whose expression is regulated by MGO treatment have been identified in MAEC endothelial cells. In particular, MGO reduces the expression of miR-190a and miR-214 both in MAEC cells and in the vascular tissue of Glo1-KD (Glyoxalase-1 Knockdown) mice, a mouse model of MGO accumulation. Gain- and loss-of-function experiments demonstrated that miR-190a and miR-214 are key mediators of MGO effects on insulin sensitivity in endothelial cells. They perform this function by regulating KRAS (Kirsten rat sarcoma viral oncogene homolog) GTPase expression, which acts upstream of ERK1/2, and the serine-phosphatase PHLPP2 (PH domain leucine-rich repeat protein phosphatase 2), which dephosphorylates

Akt in the serine residue 473, inactivating it (*Nigro et al. 2018*). MGO induced microvascular changes observed in animal models are responsible for MGO induced endothelial dysfunction and for the consequent development of DM micro and macro vascular complications.

1.4.4 MGO role in dopaminergic dysfunction and cognitive decline

The relevance of MGO and AGEs in micro and macro-vascular complications associated with DM is well assessed, but several recent studies highlighted its key role also in neurodegenerative diseases and cognitive dysfunction (*Schalkwijk & Stehouwer, 2020*). Most studies have been performed on neuronal cells in the hippocampus, a brain region essential for cognitive processes. In particular, exposure to MGO induces apoptosis by activating the mitochondrial pathway and the pathway mediated by the Fas receptor in hippocampal neurons obtained from Sprague-Dawley rats (*Di Loreto et al. 2008*). This phenomenon is accompanied by an imbalance of cytokine levels and a significant alteration of antioxidant capacity and detoxification mechanisms (*Di Loreto et al. 2008*). Similar results were obtained *ex vivo* on sections of the cerebral cortex and hippocampus taken from the brain of newborn rats. Here, MGO, following activation of ERK1/2, p38, and JNK (c-Jun N-terminal Kinase), give rise to neurotoxicity through mechanisms both dependent and independent on ROS formation (*Heimfarth et al. 2013*). Further evidence of the *in vivo* correlation between glucotoxicity and cognitive dysfunction associated with DM comes from studies conducted on various animal models and humans. It has been shown that the increase in blood glucose levels in diabetic rats treated with STZ is associated with an increase in serum MGO levels. This is accompanied by an increase in apoptosis in hippocampal neurons due to a de-regulation of the activity of the pro-apoptotic proteins caspase-3 and Bax (Bcl-2 associated X) and the anti-apoptotic protein Bcl-2 (β-cell lymphoma-2) (*Huang et al. 2012*). In Wistar rats, intracerebroventricular infusion of MGO alters GLO1 activity, increases the content of AGEs, and leads to cognitive impairment, altering the functionality of the hippocampus, but not of the frontal cortex (*Hansen et al. 2016*). Furthermore, short- and long-term memory and short-term spatial memory are impaired by intracerebroventricular injection of MGO in rats (*Lissner et al. 2021*). More recent studies show that treatment with MGO induces depressive behaviors and alters working memory in mice while significantly reducing DA and serotonin levels in the cerebral cortex (*de Almeida et al. 2021*). Finally, the importance of MGO in cognitive impairment is confirmed by the observation that aminoguanidine, a compound that inhibits

the action of MGO, prevents the onset of early cognitive deficit in rats (*Hansen et al. 2016*). A significant role of dicarbonyl stress in the cognitive decline associated with neurodegenerative diseases, such as Alzheimer's and PD, has been highlighted in humans (*Smith et al. 1995; Vicente Miranda et al. 2016*). In particular, the levels of AGEs are elevated in the cerebrospinal fluid (CSF) of Alzheimer's disease patients and MGO levels are higher in the serum of individuals with mild cognitive impairment (*Haddad et al. 2019*). On the other hand, in non-demented elderly subjects, a higher serum quantity of MGO is associated with a faster cognitive and memory decline (*West et al. 2014*). Additionally, increased serum MGO levels in human are associated with impaired memory, worst executive function, and lower gray matter volume, supporting the idea that glucotoxicity is involved in brain atrophy and cognitive dysfunction (*Srikanth et al. 2013*). Several experimental data suggest that glucotoxicity and particularly MGO can also alter the dopaminergic system (*Deng et al. 2012*). First, a neurotoxin called ADTIQ (1-acetyl-6, 7-dihydroxy-1, 2, 3, 4-tetrahydro-isoquinoline), derived from the reaction between MGO and DA, is abundant in the putamen and caudate nucleus regions of PD. It has been identified in frozen human brain tissue (*Deng et al. 2012*). ADTIQ has a structural similarity to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), known to induce symptoms of PD and acting as a neurotoxin in SH–SY5Y neuroblastoma cells (*Xie et al. 2015*). Interestingly, its levels are significantly increased in cell models under hyperglycemic conditions and in the brains of diabetic rats (*Song et al. 2014*). Therefore, the MGO-induced increase in ADTIQ levels could contribute to dopaminergic neuron dysfunction in DM (*Schalkwijk & Stehouwer, 2020*). In mice, MGO-induced alterations of the dopaminergic system have been observed. The unilateral stereotaxic administration of MGO in the *substantia nigra* leads to the formation of aggregates of SNCA accompanied by a significant reduction in TH protein levels and DJ-1 protein (*Sharma et al. 2019*). Finally, at molecular level, it has been shown that MGO regulates gene expression by modifying the expression of survival factors in rat hippocampal neurons (*Chu et al. 2016; Di Loreto et al. 2004; Di Loreto et al. 2008*) and increasing the expression of TH and the DAT in SH-SY5Y cells (*Xie et al. 2014*). Importantly, repeated intraperitoneal injection of MGO in mice reduces the amount of DA in the prefrontal cortex, inducing cognitive dysfunction (*Szczepanik et al. 2020*).

However, the impact of MGO on the molecular alterations underling DM-associated dopaminergic dysfunction is still poorly addressed.

2. AIM OF THE STUDY

Metabolic and neurodegenerative diseases have attracted attention in the last decades due to their high incidence worldwide, causing massive human and economic losses. Evidence that, among the metabolic disorders, DM appears to be strongly associated with cognitive impairment has been accumulating in recent years and a cause-effect relationship between these pathological conditions is now well-established.

DM results from a deficient insulin-dependent signaling because of low insulin levels and/or insulin resistance and it is characterized by hyperglycemia, responsible for the onset of long-term diabetic complications. Evidence in literature show that both impaired insulin signaling and hyperglycemia are sufficient to affect dopaminergic system, which is critical in controlling several neurological functions, including cognition. It is therefore proposed that alterations in DA function secondary to these metabolic dysfunctions underlie much of the cognitive impairment observed in DM. At present, however, the mechanisms behind the relative contribution of impaired insulin signaling and hyperglycemia to dopaminergic dysfunction are still unknown.

Thus, the first part of the present work aims at investigating a potential involvement of insulin in DA homeostasis, in order to give an explanation for the contribution of the impaired insulin signaling to the dopaminergic dysfunction. In this contest, the insulin-dependent regulation of the expression of TH, the rate-limiting enzyme of DA biosynthesis, has been examined. In particular, attention was focused on the identification of some transcription factors activated by insulin and involved in TH regulation in a widely used neuronal cell model (PC12 cells, pheochromocytoma cells).

The second part of the present work aims at investigating hyperglycemia impact on dopaminergic dysfunction, focusing on the potential role of MGO, a highly reactive di-carbonyl compound which is a glycolysis side-product abnormally abundant in hyperglycemic conditions. For this purpose, the expression of genes involved in DA metabolism has been examined, in presence of MGO, in a cell model of dopaminergic neurons (SH-SY5Y cells). Since in our laboratory has already been demonstrated that MGO affects miRNA levels in endothelial cells (*Mirra et al. 2017; Nigro et al. 2018*), the potential involvement of MGO-dependent miRNAs has been also evaluated.

3. MATERIALS and METHODS

***Reagents*.** Media, sera and antibiotics for cell culture were from Lonza (Walkersville, MD, USA). Protein electrophoresis and Western blot reagents were from Bio-Rad (Richmond, VA, USA) and ECL reagents LiteAblot® PLUS kit were from Euroclone (#EMP011005; Milan, Italy). The primary antibodies used were: COMT (COMT D4N6M, Cell Signaling #14368S); SNCA (αSynuclein Antibody, Cell Signaling #2642S); MAO A (MAOA Antibody, Cell Signaling #66935); MAO B (MAO-B D-6, Santa Cruz Biotechnology #sc515354); DDC (DDC D6N8N, Cell Signaling #13561); β-actin (Santa Cruz Biotechnology #sc47778); TH (monoclonal anti-Tyrosine hydroxylase clone TH-2, Sigma Aldrich #T1299); vinculin (7F9, Santa Cruz Biotechnology #sc73614); secondary antibodies were: anti-mouse (Goat anti Mouse IgG (H/L): HRP, BioRad #STAR117P); anti-rabbit (Goat anti Rabbit IgG (H/L):HRP, BioRad #STAR124P). QIAzol Lysis Reagent were from QIagen (Hilden, Germany), and SuperScript III were from Invitrogen (Carlsbad, California, USA). SYBR Green Supermix was from Bio-Rad (Hercules, California, USA). All other chemicals werefrom Sigma (St Louis, MO, USA).

Cell culture. PC12 cells were grown in T75 flasks using RPMI 1640 supplemented with 10% (v/v) horse serum (HS) and 5% fetal bovine serum (FBS) at 37°C in a 5% CO_2 humidified atmosphere. Where indicated, cells were treated with insulin (Humulin®) U-100 (Eli Lilly, Indianapolis, IN) at a final concentration of 100nM for the indicated time.
SH-SY5Y human neuroblastoma cells derived from SK-N-SH cell line isolated from the bone marrow biopsy of a 4-year-old girl with neuroblastoma. SH-SY5Y cells exhibit many characteristics of dopaminergic neurons (*Xie et al. 2010*) and, therefore, can be used as an in vitro model for neurodegenerative diseases. Cells were plated in T75 flasks using Dulbecco's modified Eagle's medium (DMEM) containing 1 g/L glucose supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 U/mL penicillin, 50 µg/mL streptomycin, 1.5 µg/mL amphotericin B (Sigma-Aldrich, Saint Louis, MO, USA). Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. Where indicated, SH-SY5Y cells were differentiated with retinoic acid (RA) 10 µM, and TPA (12-O-tetradecanoylphorbol-13-acetate) 80 nM for 6 days and then treated with 400 µM MGO for 48 hours. RA, TPA and MGO were

purchased from Sigma Aldrich (Saint Louis, Missouri, USA), product number: RA #R2625; TPA #P8139; #M0252.

Sulforhodamine B assay. The Sulforhodamine B viability assay measures cytotoxicity and cell proliferation and is therefore used for extensive drug or molecule screening. The assay is based on the ability of the dye sulforhodamine B to bind the basic amino acid residues of cell proteins fixed with Trichloroacetic Acid (TCA). The assay results are proportional to the number of cells and intracellular proteins. The assay provides colorimetric detection, is not dangerous to cells, and is stable over the long term.
Approximately 10,000 cells per well of 96-plate were plated and treated for 24h, 48h and 72h with MGO 200µM, 400µM, and 600µM. At the end of the incubation, 25µL of cold TCA (50% in H2O) was added per well, and the plates were stirred for 1h at 4 ° C to fix the proteins. Three washes were then carried out with distilled water, and then 50µL of Sulforhodamine B 0.4% in 1% Acetic Acid were added. After 30 minutes of incubation at room temperature, three washes with 1% acetic acid were performed, and the inverted plates were left to dry overnight. Then, 100µL of 10mM Tris-HCl at pH 7.5 was added, and the plate was stirred for 30 minutes to solubilize the lumps. The results were read to the Glomax reader at an absorbance of 495nm.

Cell transfection. PC12 cells were transietly transfected with a dominant negative version of HIF-1α that lacks both binding and transactivation domains (pcDN3-HA-DN-HIF-1α, kindly gifted from Dr, Jacques Pouyssegur), with a deletion mutant of Nurr77 obtained by its amino acid 1-574 fragments (pCMV-FLAG-Nurr77-dAF-2, kindly gifted from Prof. Makoto Makishima), and with the corresponding empty vector (EVs). The transfection were performed using a Microporator (Invitrogen, Waltham, Massachusetts, USA) and following the manufacturer's instructions.
SH-SY5Y were seeded in 60-mm plates. miR-190a and miR-214 levels were modulated by transient transfection of previously differentiated SH-SY5Y cells with specific mimics and antagomiRs (antimiRs). Concentration used of miR-190a: 5 mmol/L of miR-190a mimic (mmu- miR-190a miRIDIAN Mimic) and 50 mmol/L of the miR-190a-inhibitor (mmu-miR-190a miRIDIAN Hairpin Inhibitor). Levels of miR-214 were also modulated by transient transfection with 50 mmol/L of miR-214 mimic (mmu-miR-214 miRIDIAN Mimic) and 5 mmol/L of miR-214-inhibitor (mmu-miR-214 miRIDIAN Hairpin Inhibitor).

The miRIDIAN miRNA Mimic negative control #1 and miRIDIAN miRNA Hairpin Inhibitor negative control #1 (Dharmacon, Lafaytte, CO, USA) were used as miRNA-mimic and miRNA-antimiR negative controls, respectively. Cells were transfected using Lipofectamine^{TM} 3000 (Invitrogen, Waltham, Massachusetts, USA) reagent, following the manufacturer's instructions. 48 hours after transfection, the cells were harvested and treated as described below.

Western blot assay. PC12 cells were solubilized in lysis buffer (50mM Hepes, pH 7.5; 150mM NaCl; 10mM EDTA; 10mM $Na_2P_2O_7$; 2mM Na_3VO_4; 100mM NaF; 10% glycerol; 1% Triton X-100; 1 mM phenylmethylsulfonylfluoride and 10 µg/mL aprotinin) for 2h at 4°C.

SH-SY5Y cells were solubilized in RIPA lysis buffer (50mM Tris-HCl, pH 7.4; 150mM NaCl; 1mM EDTA; 1mM EGTA; 1% NP-40; 0.5% deoxycholic acid $C_{24}H_{40}O_4$; 0.1% SDS; 1 mM phenylmethylsulfonylfluoride and 10 µg/mL aprotinin) and subjected to thermal shock: 30 minutes at 4°C followed by 2 hours at -80°C.

Cell lysates were clarified by centrifugation at 13200 rpm for 20 minutes. Proteins in the supernatant were assayed using the Bradford color assay. 50µg of cell lysates were then separated by SDS-PAGE and transferred to 0.2µm Immobilon-P PVDF membranes (Merck KGaA, Darmstadt, Germany). Upon incubation with primary and secondary antibodies, immmunoreactive bands were detected by chemiluminescence. Densitometric analysis was performed using ImageJ software.

RNA isolation. Total RNA was isolated from SH-SY5Y and PC12 cells using QIazol reagent (QIagen) that allows RNA isolation, exploiting the principle of separation phase and precipitation of nucleic acids with alcohols. In particular, cells were collected in 500 µL of QIazol and lysed by shake with vortex mixer to facilitate disintegration of membranes. After that, 100 µL of chloroform was added and mixed; then, the samples were incubated for 3 minutes at room temperature. Subsequently, samples were centrifuged at 13200 rpm for 10 minutes at 4 °C. At this point, the samples are separated in three phases: an organic-phenolic lower phase, an interphase consisting of cellular debris and an upper aqueous phase containing RNA. The aqueous phase was recovered and the RNA was precipitated by adding 250 µL of cold isopropanol followed by centrifugation at 13.200 rpm for 20 minutes at 4 °C. Subsequently, the pellets were washed with 250 µL of 70%

cold ethanol and centrifuged at 7500 g for 5 minutes at 4 °C. The supernatants were completely removed and the RNA pellets resuspended in 30 µL of micro filtered distilled water.

RNA quantification. RNA concentration was measured by spectrophotometric method which exploits the properties of nucleic acids to absorb UV light with an absorption peak at a wavelength of 260 nm. To evaluate RNA concentration was used MaestroNano Pro (MN-913) spectrophotometer (MaestroGen Inc, Hsinchu City, Taiwan). RNA concentration is read by loading 2 µL of sample on the MaestroNano optical plate and an equal amount of solvent (micro-filtered distilled water) has been read as white.

Reverse transcription. 1 µg of total RNA was reverse transcribed in cDNA using SuperScript® III Reverse Transcriptase (Invitrogen Life Technologies, CA, USA). The samples were reverse transcribed into a final reaction volume of 20 µL using the T3000 Thermocycler (Biometra®) and the following protocol: 5 minutes at 65 °C, 10 minutes at 25°C, 1 hour at 42 °C, 15 minutes at 70 °C. The reaction mix consists of:

- Mix 1: 10 ng/µL random primer and 5 mM deoxy-nucleotide triphosphate (dNTP).
- Mix 2: 5X Buffer, 0.1 M dithiotritol (DTT) and 100 U Reverse Transcriptase Polymerase (RTpol).

miRNAs evaluation: total RNA were reverse transcribed in cDNA using the miScript II RT kit (Qiagen Science, Germany) and the T3000 Thermocycler (Biometra®) following the protocol: 60 minutes at 37°C, 5 minutes at 95°C. The reaction mix consists of: 5x miScript HiSpec Buffer, 10x miScript Nucleics Mix, miScript Reverse Transcriptase Mix.

Real Time-PCR. For quantitative analysis of gene expression, 25 ng of cDNA was analyzed by Real Time PCR using 2X iQ™ SYBR® Green supermix (Biorad, CA) and the reaction was performed in the "Bio-Rad iCycler iQ5 Real Time PCR" thermocycler. For each gene analyzed a reaction mix was prepared. The mix is composed by 10 µM forward and reverse primers, iQ™ SYBR® Green supermix 2X and micro filtered distilled water added for a final volume of 10 µL. All reactions were performed in triplicate using the β-actin and 18S rRNA genes as internal controls. Specific primers used for amplification were purchased from QIAGEN (Hilden, Germany) and listed below:

18S rRNA	F: 5’ – GGTGGTGCATGGCCGTTCTTAGTT – 3’ R: 5’ – CTGAACGCCACTTGTCCCTCTAA – 3’
TH	F: 5’ – CCAGCTTCTGGAACGGTACT – 3’ R: 5’ – GACTGGCCAGAAAATCACG – 3’
Nurr77	F: 5’ – GCACAGCTTGGGTGTTGATG – 3’ R: 5’ – ACAGCTAGCAATGCGGTTCT – 3’
β-actin	F: 5’ – AAGATCAAGATCATTGCTCCTCCTG – 3’ R: 5’ – AGCTCAGTAACAGTCCGCCT – 3’
h TH	F: 5’ – TTCCTGAAGGAGCGCACG – 3’ R: 5’ – CGTGGCGGATATACTGGGTG – 3’
h DDC	F: 5’ – GCAATCAATGTTCACGCAAC – 3’ R: 5’ – AGGCATTTAGCCACATGACAA – 3’
h MAO a	F: 5’ – CTGATCGACTTGCTAAGCTAC – 3’ R: 5’ – ATGCACTGGATGTAAAGCTTC – 3’
h MAO b	F: 5’ – GCTTCTCTGGTTCCTGTGGTATGTG – 3’ R: 5’ – TCCGCTCACTCACTTGACCAGATC – 3’
h COMT	F: 5’ – TCCTAAATGCAAAGCACACC – 3’ R: 5’- CAATCCAGTGTTGCAGTTCAG – 3’
h SNCA	F: 5’ – GTGGCTGCTGCTGAGAAAAC – 3’ R: 5’ – TCTTCTCAGCCACTGTTGCC – 3’
h BDNF	F: 5’ – CTCTGGAGAGCGTGAATGGG – 3’ R: 5’ – CGTGTACAAGTCTGCGTCCT – 3’
GAP 43	F: 5’ – CACTGATAACTCGCCGTCCT – 3’ R: 5’ – CTACAGCTTCTATGTTCTTTCAGC – 3’

For miRNAs, PCR reaction mixes were analyzed using a SYBR Green 2x QuantiTect Master Mix (Qiagen Sciences, Germany). In addition, a 10x miScript Universal Primer (Qiagen Sciences, Germany) and specific primers for miRNAs were added to the reaction. Reactions were performed using the iCycler IQ system (Biorad, CA). All reactions were performed in triplicate using SNORD68 (Small nucleolar RNA) as internal control.
Qiagen company provided the specific primers used:

Hs_SNORD68_11 miScript Primer Assay, MS00033712
Mm_miR-190_3 miScript Primer Assay, MS00032438
Mm_miR-214_2 miScript Primer Assay, MS00032571
hsa-miR-7-5p: Hs_miR-7_2 miScript Primer Assay, MS00032116
hsa-miR-153-3p: Hs_miR-153_1 miScript Primer Assay, MS00008771

The values reported in graphs were obtained by the relative quantification method, in which the threshold cycles (Ct) of the genes analyzed (Ctx) are normalized on the Ct of the gene used as normalizer. In more detail, the calculation uses the following formula:

VALUE = $e^{-\Delta\Delta Ct}$,

where

ΔΔCt = ΔCt SAMPLE - ΔCt CONTROL

ΔCtx = CtX – Ct NORMALIZER

Chromatin Immunoprecipitation (ChIP) Assay. ChIP assays were performed as previously reported by *Ungaro et al. 2008*. Upon protein-DNA cross-linking, cells were lysed and sonicated to have chromatin fragments of about 500-1000 bp in size. Lysates were incubated over-night with specific HIF-1α or Nurr77 antibodies (Santa Cruz Biotechnology) and the complexes were isolated using protein A-agarose/salmon sperm DNA, purchased from Millipore. As negative control, a non specific rabbit IgG antibody was used. Immunoprecipitates were washed more time and then eluted by freshly prepared 1% SDS, 0.1m $NaHCO_3$ buffer. After cross-linking reversion, immunoprecipitated DNA was purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and detected by real-time PCR. Fold enrichment method was used to normalize the real-time PCR data. Specific primers to HIF-1α and Nurr77 binding sites in the TH promoter were used and listed below:

hypoxia-responsive element site	F: 5' – TCTAGCGGTCTCCTGTCCCACAG – 3' R: 5' – GCGACGAGAAAGGCCTCCGTC – 3'
NurRE-like site	F: 5' – GAGATCCTGTTTCCCTGGCT – 3' R: 5' – TCAGCTGGTCCCCATGTAAG – 3'

Dopamine ELISA. The technique used to measure intracellular DA is the competitive ELISA (enzyme-linked immunosorbent assay). 100µL of the sample were analyzed using a kit supplied by LDN diagnostics (BA E-5300R). All reactions were performed in duplicate, following manufacturer's instructions. The plate was read by a GloMax reader at an absorbance of 450nm. The quantity in ng/mL of DA present in the samples of interest was obtained by interpolation based on a standard curve created using known concentration of DA solutions supplied by the kit. Each value obtained was normalized relating it to the total protein content of the corresponding sample. Bradford's colorimetric assay determined this protein content.

Statistical procedures. Data are expressed as mean ± Standard Deviation (SD) and mean ± Standard Error of the Mean (SEM) of at least three different experiments. Comparison between groups was calculated using Student's *t*-test; values with $p \leq 0.05$ were considered statistically significant.

4. RESULTS AND DISCUSSION

SECTION 1

4.1 The role of insulin in the regulation of TH expression

TH catalyzes the conversion of tyrosine in L-DOPA, which represents the rate-limiting step in DA synthesis (*Tekin et al. 2014*). TH expression is finely modulated at both the transcriptional and posttranscriptional level. Previous works have already identified several different transcription factors involved in the regulation of TH gene expression, including hypoxia-inducible factor 1-alpha (HIF-1α; *Norris & Millhorn, 1995*), activating protein-1 (AP1) and AP2, specificity protein 1 (Sp1; *Nakashima et al. 2003*) and cAMP-response Element-binding protein (*Lewis-Tuffin et al. 2004*). Interestingly, it has been demonstrated that insulin affects the subcellular localization, stability and transactivation potential of some of these transcription factors (*Solomon et al. 2008; Ke & Costa, 2006; Treins et al. 2002*). In addition, it has been shown that impaired insulin signaling is associated with alterations of TH expression and/or activity (*Glanville & Anderson, 1986; Li Y, Song YH et al. 2009; Morris et al. 2011; Nascimento et al. 2011*). Taken together, these data suggest that insulin could be able to affect DA levels regulating TH gene expression.

To better clarify the molecular basis of insulin action on TH expression, PC12 cells were used, since they are widely considered as a suitable model system for dopaminergic neurons (*Alberio et al. 2012*). After verifying the response of PC12 cells to 100nM insulin by checking the insulin induced activation of both PKB and ERK1/2 (data not shown), time course experiments were performed in PC12 cells treated with 100nM insulin and both total RNA and protein were extracted to evaluate TH mRNA and protein levels. Within 2h stimulation with insulin, TH mRNA levels increased more than 1.7-fold compared to control cells (at time zero). Upon 6h in the presence of insulin TH levels were back to the basal. Interestingly, 16h stimulation with insulin led to a second pick for TH mRNA levels, which persisted for up to 24h. TH protein levels featured a similar pattern of TH mRNA levels (Figure 6a and 6c). Then, the specific role of insulin receptor (IR) in insulin-dependent regulation of TH gene expression was confirmed. Indeed, insulin-dependent effect on TH gene expression was abolished in PC12 cells transiently transfected with a tyrosine kinase-deficient mutant IR (data not shown), obtained by the substitution of the three tyrosines in the regulatory loop (Tyr1146, Tyr1150, and Tyr1151) with phenylalanine (*Formisano et al. 2000*).

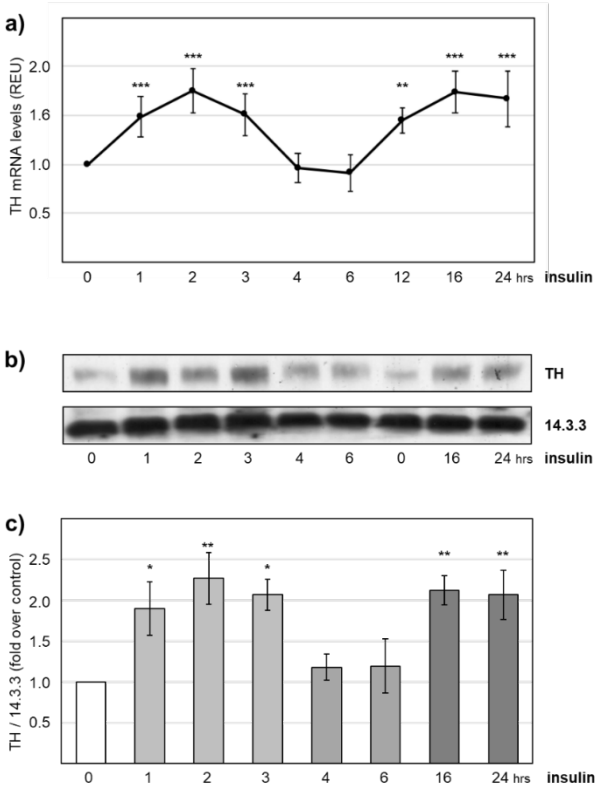

Figure 6: Insulin-dependent regulation of TH espression. PC12 cells were stimulated with insulin 100nM for different time points and TH mRNA levels were evaluated by real-time PCR using 18s rRNA as a reference gene to normalize TH. Results are shown as mean ± SD of more tha three indipendent experiments (a). Th protein levels were evaluated by Western blot using 14.3.3 as a loading control. A representative blot (b) and th densitometric analysis (c) of three indipendent experiments are shown in the figure. Bars in the graph represent the mean ± SD, expressed as fold over control. For all the results shown in the figure, statistical analysis was evaluated using Student's t test (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). mRNA: messenger RNA; PCR: polymerase chain reaction; rRNA: ribosomal RNA; SD: standard deviation; TH: tyrosine hydroxylase

Furthermore, it was found that both PI3K and ERK1/2 were involved in the regulation of TH expression by insulin. Indeed, insulin effect on TH expression was abolished in presence of specific pharmacological inhibitors of PI3K or ERK1/2 (*Bartella et al. 2008; Dimova & Kietzmann, 2006; Kietzmann et al. 2003; Liu LZ et al. 2011; Treins et al. 2002*) (data not shown).

4.2 The role of HIF-1α in the regulation of TH expression by insulin

Once demonstrated that TH transcription is insulin-dependent, the following step was to investigate the role of transcription factors depending on insulin action and potentially involved in TH regulation. It is well known that TH transcription is tightly regulated by a plethora of transcription factors (*Tekin et al. 2014*). Among these, HIF-1α represented a promising candidate. Indeed, HIF-1α is activated by insulin in several cell types, it acts downstream PI3K and ERK1/2 (*Liu LZ et al. 2011; Zhao et al. 2014; Bartella et al. 2008;*

Dimova & Kietzmann, 2006; Kietzmann et al. 2003; Treins et al. 2002), and it binds a hypoxia-responsive element (HRE) in TH promoter, contributing to TH regulation (*Norris & Millhorn, 1995*). Interestingly, upon stimulation with 100nM insulin for 2h and 16h, a significant increase in HIF-1α protein levels was found in PC12 cells (data not shown). To investigate the molecular mechanisms underlying HIF-1α involvement, ChIP assays were performed. Interestingly, the binding of HIF-1α to TH promoter increases by fourfold upon 2h stimulation with 100 nM insulin. No increase of HIF-1α binding to TH promoter was observed upon 6h insulin stimulation and only a slight increase by 1.5-fold was observed upon 16h insulin stimulation (Figure 7).

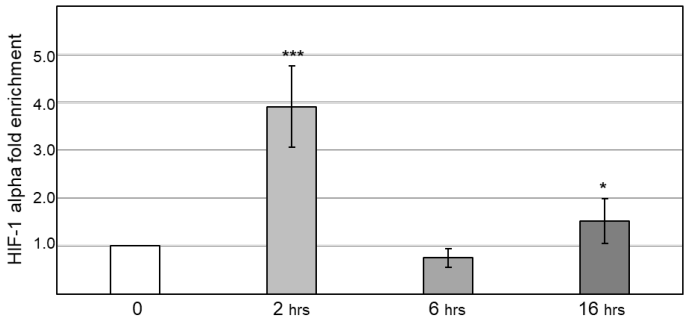

Figure 7: HIF-1α binding to TH promoter. PC12 cells were stimulated with insulin 100nM for 2, 6, or 16h and the binding of HIF-1α to TH promoter was evaluated by ChIP assays. Soluble chromatin was prepared and immunoprecipitated with antibodies specific for HIF-1α, or immunoprecipitated with an IgG antibody as a negative control. Purified DNA was amplified by real-time PCR with primers specific for the HRE site at the rat TH ptomoter. Bars represent the mean ± SD of three indipendent experiments. Statistical analysis was evaluated using Student's t test (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). ChIP: chromatin immunoprecipitation; HIF-1α: hypoxia-inducible factor 1-alpha; HRE: hypoxia-responsive element; PCR: polymerase chain reaction; SD: standard deviation; TH: tyrosine hydroxylase

However, the transient transfection of PC12 cells with a HIF-1α dominant negative construct (DN_HIF-1α; *Richard et al. 2000*) allowed to confirm the key role of HIF-1α in TH regulation by insulin upon both 2h and 16h stimulation (Figure 8a).

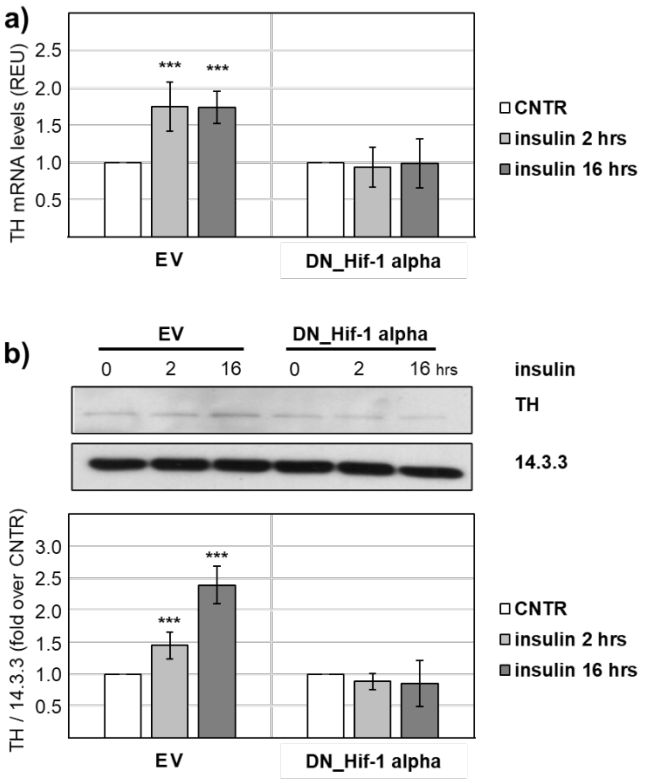

Figure 8: Role of HIF-1 in the regulation of TH espression. PC12 cells were transiently transfected with a dominant negative of HIF-1α (DN_HIF-1α) or an empty vector (EV) and then stimulated with insulin 100nM for 2h or 16h or not (CNTR). TH mRNA levels were quantified by real-time PCR using 18s rRNA as a reference gene. Bars represent the mean ± SD of three independent experiments (a). Levels of TH protein were evaluated by western blot in the transfected PC12 cells, treated with insulin or not (CNTR). 14.3.3 was used as a loading control. A representative blot and the densitometric analysis of the protein levels of three independent experiments are shown in the figure. Bars in the graphs represent the mean ± SD, expressed as fold over control (b). For all the results shown in the figure, statistical analysis was evaluated using Student's t test (*$p \leq 0.05$, ***$p \leq 0.001$). HIF-1α: hypoxia-inducible factor 1-alpha; mRNA: messenger RNA; PCR: polymerase chain reaction; rRNA: ribosomal RNA; SD: standard deviation; TH: tyrosine hydroxylase

Therefore, the HIF-1α-dependent effect on insulin-induced TH regulation observed after 16h insulin stimulation, when HIF-1α presence at TH promoter is not relevant, suggested that HIF-1α has an indirect action in the late effect due to insulin stimulation (Figure 8b).

These results prompted the idea that the late effect of insulin was mediated by a different transcription factor which was under the regulation of HIF-1α.

4.3 The role of Nur77 in the regulation of TH expression by insulin

Several transcription factors are demonstrated to be regulated by HIF-1α, such as nerve growth factor IB, also known as Nur77 or NR4A1 (*Choi et al. 2004*), ETS proto-oncogene 1 (Ets1; *Oikawa et al. 2001*), differentiated embryo-chondrocyte expressed gene 1 (Dec1) and basic-helix-loop-helix family member e41 (Dec2; *Duren et al. 2016; Miyazaki et al. 2002*). Thus, the expression levels of these transcription factors were evaluated in PC12

cells stimulated with insulin and solely the expression of Nur77 resulted to be regulated by insulin. In particular, Nur77 mRNA levels were increased 1.5-fold and 3.5-fold upon 2h and 16h insulin stimulation respectively (data not shown). Interestingly, in the presence of the HIF-1α dominant negative construct, insulin was not able to increase Nur77 mRNA and protein levels (data not shown). To verify the presence of Nur77 on TH promoter, ChIP assays were performed. As shown in Figure 9a**,** the binding of Nur77 to TH promoter increases by twofold upon 2h insulin stimulation and by up to fourfold upon 16h insulin stimulation. Finally, to better clarify the key role of Nur77 in the regulation of TH expression by insulin, PC12 cells were transiently transfected with a Nur77 dominant negative construct (DN_Nur77; *Ishizawa et al. 2012*). Interestingly, insulin ability to increase TH mRNA levels upon 16h incubation was abolished in presence of DN_Nur77 (Figure 9b). These findings confirm that HIF-1α mediates the late effect of insulin on TH expression via Nur77.

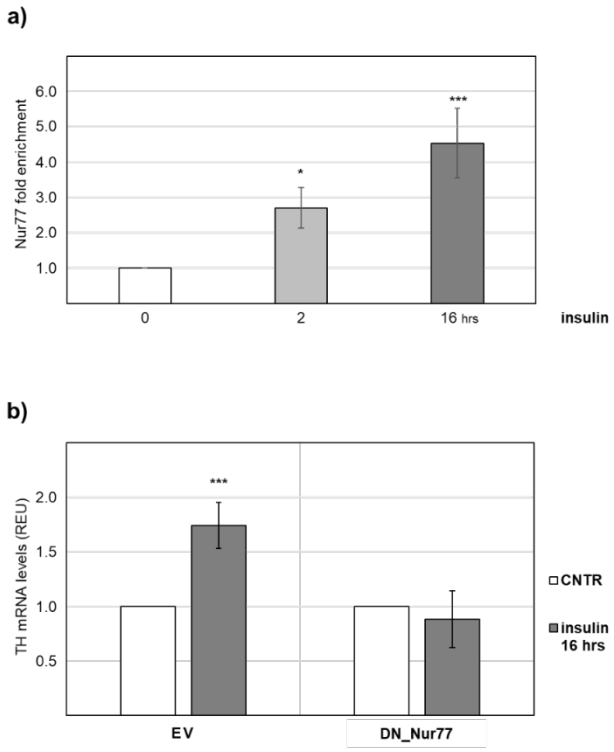

Figure 9: Role of Nurr77 in the regulation of TH expression. PC12 cells were stimulated or not with insulin 100nM for 2h or 16h and the binding of Nurr77 to TH promoter was evaluated by ChIP assays. Soluble chromatin was prepared and immunoprecipitated with an antibody specific for Nurr77 or with an IgG antibody as a negative control. Purified DNA was amplified by real-time PCR with primers specific for the NurRE-like site at the rat TH promoter. Bars represent the mean ± SD of three indipendent experiments (a). PC12 cells were transiently transfected with a dominant negative of Nurr77 (DN_Nur77) or with an empty vector (EV) and then stimulated with insulin 100nM for 16h or not (CNTR). TH mRNA levels were quantified by real-time PCR using 18s rRNA as a reference gene to normalize TH. Bars represent the mean ± SD of three indipendent experiments (b). For all the results shown in the figure, statistical analysis was evaluated using Student's t test (*$p \leq 0.05$, ***$p \leq 0.001$). ChIP: chromatin immuniprecipitation; mRNA: messenger RNA; PCR: polymerase chain reaction; rRNA: ribosomal RNA; SD: standard deviation; TH: tyrosine hydroxylase

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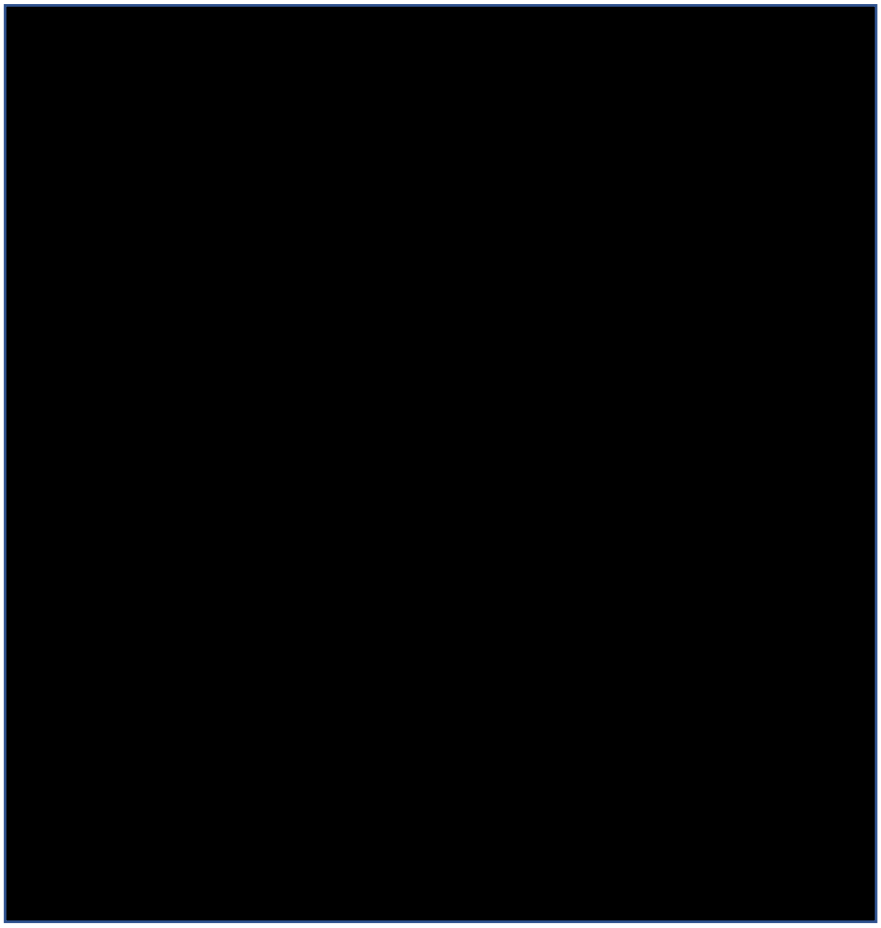

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6. APPENDIX: ***microRNAs***

MicroRNAs (miRNAs) are small, single-stranded, non-coding RNA molecules 20-25 nucleotides long, highly conserved in species and produced by all cells in the body. They play a significant role in RNA silencing and post-transcriptional regulation of gene expression esploiting the complementary sequences present on target mRNA molecules (*Mirra et al. 2018*).

According to the latest version (version 22) of the official miRNAs database, known as miRBase, to date 48.885 mature miRNAs have been identified from 271 organisms, and it has been estimated that they regulate 60% of genes present in the entire genome. They control several different biological processes, such as embryonic development, cell differentiation, cell growth control, apoptosis, and metabolic processes (*He & Hannon, 2004*). Therefore, it is not surprising that alterations in miRNAs levels contribute to pathological conditions.

The biogenesis of mature miRNAs is a complex process that originates in the nucleus and ends in the cytoplasm. Transcription of miRNA genes is predominantly mediated by RNA polymerase II (Pol II) (*Lee et al. 2004; Cai et al. 2004*), although there are cases of transcription by RNA polymerase III (Pol III) (*Borchert, 2006*). Genes encoding miRNAs are evolutionarily well conserved and located in introns or exons of protein-encoding genes and intragenic areas. miRNAs are encoded as long primary transcripts, indicated as pri-miRNA (Primary miRNA), which fold to form a partial double helix, characterized by one or more hairpin folds (stem-loops). In the nucleus, miRNAs undergo a first cleavage reaction leading to the formation of pre-miRNAs (approximately 70-100 nucleotides long) and characterized by the peculiar protrusion of two nucleotides at the 3'-ends (*Lee et al. 2003*). This asymmetric end is important in the subsequent step of maturation in the cytoplasm, where a type II RNA-endonuclease allows the transition from pre-miRNA to double-stranded (duplex) RNA molecules (about 20-22 nucleotides long), consisting of the mature miRNA strand and the complementary strand, known as miRNA-star (*Schwarz et al. 2003*). Those strands of the duplex with lower stability at the 5'-end usually became mature miRNAs, recognized by the multiprotein complex RISC (RNA induced-silencing complex), which selects them as active miRNAs (*Bartel, 2004*) and addresses them towards the mRNAs target where they exert their repressive action.

On the other hand, the complementary miRNA-star strand is rapidly degraded by a helicase present in the RISC complex (*Schwarz et al. 2003*). Therefore, the thermodynamic properties of miRNAs determine their assembly with RISC and, consequently, the specificity for mRNA target. However, if both 5'-ends of the duplex show comparable stability, both strands can be selected as mature miRNAs with biological activity (*He & Hannon, 2004*).
Once incorporated into the RISC complex miRNAs can control gene expression at the post-transcriptional level in two ways:

- *Translation repression*: defect in the association of mRNA target with the ribosome; blocking or slowing down of the translation elongation phases; premature separation of the ribosome and/or proteolysis of the nascent peptide (*Maroney et al. 2006; Humphreys et al. 2005*);
- *Degradation of mRNA target*: through de-adenylation or decapping mechanisms. miRNAs cause the sequestration of mRNAs in the so-called processing bodies, known as P-bodies (cytoplasmic loci enriched with factors involved in the degradation of mRNAs) (*Eulalio et al. 2008*).

The mode of miRNAs repression depends on the complementarity between a region of 6-8 nucleotides located at the 5'-end of the miRNA, called seed region, and the 3'-UTR of the mRNA (*Bartel, 2004*), which may contain multiple binding sites for miRNAs. However, some studies identified miRNA binding sites also in the 5'-UTR regions or in coding regions (*Duursma et al. 2008*). The grade of miRNA-mRNA complementarity is considered a key element in the regulatory mechanism of miRNA-mediated silencing. In the case of perfect complementarity, degradation of the mRNA target occurs; instead, in the case of imperfect pairing, there is only inhibition of the translation of the mRNA target. The final effect of this last negative control is the stop of translation and the mRNA transport in the P-bodies (*Kulkarni et al. 2010*). Furthermore, from a functional point of view, each miRNA can recognize multiple targets, and multiple miRNAs can potentially regulate the same target.

The regulation of miRNA expression is essential for their role in biological processes, therefore it is regulated at several different levels (transcription, processing, editing, miRNA degradation) during their biogenesis. The transcriptional regulation of miRNAs is the same as that of protein-coding genes, since the same regulatory elements are present at

the miRNA promoter. An alteration in miRNA levels may be due, for example, to the presence of regulatory proteins that affect their biogenesis or to changes in the efficiency of nuclear transport. The presence of SNPs within the gene sequence can also influence the maturation of the transcript and the binding of miRNAs to target messengers; for example, the substitution of guanine with a cytosine seems to disadvantage silencing. Furthermore, the function of miRNAs can also be influenced by RNA-binding proteins (RBP) that can associate with mRNA and prevent its binding with the miRNAs or interact with miRNA precursors (*Jazdzewski et al. 2008*).

Regarding the role of miRNAs in metabolism, more and more studies demonstrate their importance. Especially, approximately 80% of human mRNAs are estimated to be targets of miRNAs. However, to date, only a small number of miRNAs is known to be able to mediate adipocyte differentiation and function (*Arner & Kulyté, 2015; Brandão et al. 2017*), cell homeostasis and function (*Asli et al. 2008*), to controll pancreatic β-cell mass and insulin secretion (*Dumortier et al. 2016; Martinez-Sanchez et al. 2017*), and to modulate key components of insulin signal transduction (*Chakraborty et al. 2014*) in insulin-sensitive tissues. Recent studies confirm that an altered expression of miRNAs corresponds to the loss of glycemic homeostasis and the onset of pathological conditions (*Feng et al. 2016; Hashimoto & Tanaka, 2017*). The involvement of miRNAs in the pathogenesis of diabetes has been demonstrated, and the possible use of miRNAs as blood biomarkers to prevent the onset of diabetes has also been described (*Guay et al. 2011*).
DM compromises the functionality of many organs and tissues, such as the heart, kidneys, and eye, following the onset of vasculopathy and peripheral neuropathy. MiRNAs are involved in many of these complications.

- The association between the altered expression of miR-133 and cardiac hypertrophy was found in the heart muscle (*Carè et al. 2007*). Instead, alterations in miR-320 levels in myocardial microvascular endothelial cells (MMVECs) are critical since the increased expression of this miRNA is accompanied by reduced cell proliferation and migration (*Wang et al. 2009*).
- The role of miR-221 in DM-induced endothelial dysfunction in human umbilical endothelial cells (HUVECs) was investigated; in particular, its involvement in the regulation of angiogenesis was demonstrated (*Li Y, Song YH et al. 2009*). On the other hand, miR-126 is the only miRNA that seems to be specifically expressed in endothelial cells. This is involved in vascular development and angiogenesis and plays an important

role in cardiovascular pathologies and tumorigenesis (*Fernández-Hernando & Suárez, 2018*).

- In the kidney of diabetic subjects, miR-192 levels are increased by TGF-β and play a key role in developing diabetic nephropathy and renal function (*Kato et al. 2007*).
- In the eye, the overexpression of specific miRNAs in response to NF-kB, VEGF, and p53 represents an early indicator of the onset of pathological changes in diabetic retinopathy (*Kovacs et al. 2011*). Among these, the expression of miR-146, miR-155, miR-132, and miR-21 is upregulated by NF-kB in the endothelial cells of the retina of diabetic rats.

Interestingly, miRNAs also play a central role in cognitive decline. It has been observed that in subjects with cognitive impairments, miRNA levels are abnormal in both the brain and body fluids. In particular,

- miR-34 is altered in the prefrontal cortex of schizophrenic patients (*Chen et al. 2021*);
- miR-9, miR-125b, miR-146, and miR-155 are upregulated in the cerebrospinal fluid of AD patients, and their expression is regulated by NF-kB signaling (*Alexandrov et al. 2012; Denk et al. 2015*);
- miR-132 and miR-134 appeared as potential biomarkers of early dementia. These miRNAs are altered in both subjects with mild cognitive impairment (MCI) and subjects with AD. Furthermore, their dysregulation can be used to predict MCI with high sensitivity and specificity, even five years before the actual clinical diagnosis (*Sheinerman et al. 2013*).

Thus, the key role of miRNAs in post-transcriptional gene regulation in lipid and glucose metabolism, in vascular DM-associated complications, suggests the potential use of these molecules as biological markers and targets of new drugs for the prevention and treatment of DM and its complications.

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