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## Fibroblast Growth Factor-21 (FGF-21) enhances mitochondrial functions and increases the activity of PGC-1α in human dopaminergic neurons.

## Il Fattore di crescita FGF-21 potenzia le funzioni mitocondriali ed incrementa l'attività di PGC-1α in neuroni dopaminergici umani.

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# ***Summary***

The Fibroblast Growth factor 21 (FGF-21) is a member of the FGFs family and forms a subfamily with FGF-19 and FGF-23. These three unique FGFs are able to function in an endocrine manner. FGF-21 plays important roles in metabolic regulations including, glucose homeostasis, energy expenditure, and lipid metabolism; it is widely express in metabolic tissues such as liver, adipose tissue, muscle, and pancreas. Thus FGF-21 has been largely studied due to its physiological functions, but very little is known yet about the expression of FGF-21 in the brain, and its possible actions in the Central Nervous System (CNS). FGF-21 exerts also its functions through the involvement of other factors, modulating the expression of specific proteins, that activate downstream pathways in response to certain stimuli. One of the main target of FGF-21 is the Peroxisome proliferator-activated receptor co-activator protein-1α (PGC-1α), a transcriptional co-activator that is increased in several tissues by FGF-21. PGC-1α is a major regulator of mitochondrial functions, biogenesis, and antioxidant response and it was suggested as a therapeutic target in Parkinson's Disease (PD). According to this, recently Mudò et al. have demonstrate, the neuroprotective effect of PGC-1α, due to its antioxidant actions, in an animal model of PD. PGC-1α is regulated by both transcriptional and posttranslational events, as deacetylation of specific lysine residues performed by specific deacetilase like SIRT-1. These transcriptional and posttranslational modifications involve in addiction the action of growth factors. Therefore, on the basis of these observations, using different approaches, the aim of present study was to investigate the possible interactions between the growth factor FGF-21 and PGC-1α in dopaminergic neurons, and its possible expression in the brain. We demonstrate here that FGF-21 increases the levels of PGC-1 α and its transcription, enhancing the gene promoter activity in human dopaminergic cell culture. Moreover in these cells FGF-21 is able to increase the activity of PGC-1α and elevate mitochondrial antioxidants. The activation of PGC-1α by FGF-21 occurs via the $NAD^+$-dependent deacetylase SIRT-1 subsequent to an increase in the enzyme nicotinamide phosphoribosyltransferase (Nampt), that is the is the rate-limiting enzyme in $NAD^+$ biosynthesis. The increase of mitochondrial antioxidant enzymes levels is accompanied by enhanced mitochondrial respiratory capacity, as shown in real-time analyses of living cells, but not by an increase of the number of these organelles in FGF-21 treated cells. Our results suggest that

FGF-21 enhances in human dopaminergic neurons, the mitochondrial activity, by inducing the increase of levels and activity of PGC-1α, via SIRT-1. In vivo, to investigate if FGF-21 was locally produced in the brain, we asses FGF-21 levels in some brain areas using immunoblot analysis. We observed that FGF-21 is expressed in several brain regions, such as the cortex, hippocampus, striatum and substantia nigra. Next, in order to identify brain cells type involved in FFG-21 production , we performed immunohistochemistry assays, using substantia nigra sections from adult rats brain treated with Kainic acid, that induces an excitotoxic damage. These assays reveal that neither the dopaminergic neurons nor astrocytes are involved in the FGF-21 production, microglia cells rather express the growth factor, suggesting a role of FGF-21 in the neuroimmunological response.

Taken together these results show that dopaminergic neurons respond to FGF-21 by enhancing the mitochondrial capacity and altering gene pathways regulated by PGC-1α, and that FGF-21 is expressed in the brain by microglial cells, probably following a damage that can active a neuroimmunological response.

These findings can open the way to study the potential benefits of FGF-21 treatment in different neurological disorders such as in Parkinson's Disease

# Background

## Fibroblast Growth Factor 21

Fibroblast Growth Factor 21 (FGF-21) was first isolated from mouse embryo DNA, its murine gene is located in chromosome 7, while human FGF-21 gene is located in chromosome 19 (Nishimura T. et al, 2000; Homo Sapiens Gene ID 2012). FGF-21 is a 209-amino acid-long protein with an N-terminal signaling peptide, that after the cleavage, results a mature protein with 181 amino acid (w20 kDa) (Huating L. et al, 2013); it is classified as a Fibroblast Growth Factor based on its structure, because it contains a common domains and shares 10%–30% sequence identity with other Fibroblast Growth Factors.

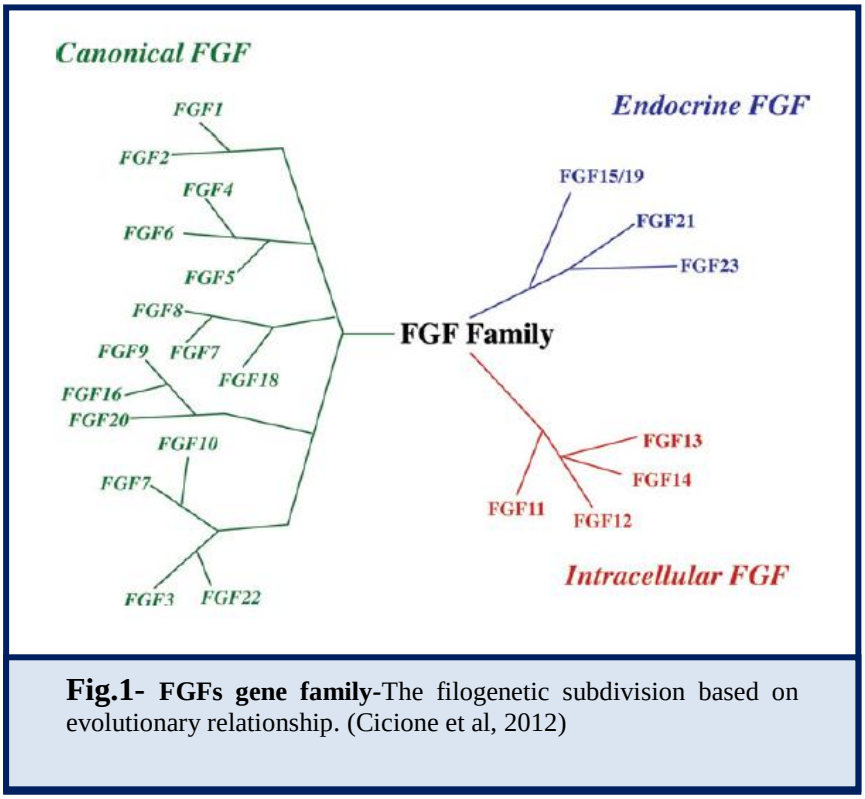


**Fig.1- FGFs gene family**-The filogenetic subdivision based on evolutionary relationship. (Cicione et al, 2012)

The Fibroblast Growth Factor superfamily, named by its ability to stimulate fibroblast proliferation, contains 22 proteins, divided into subfamilies, based on their structural similarities and modes of action. The Gene family can be divided into three subfamilies: the *intracellular* FGFs (FGF11/12/13/14), the *endocrine* FGFs (FGF15/19/ 21/23), and the *paracrine* FGFs (others) (Long Y. et al, 2011; Itoh and Ornitz, 2008; Kuro-o, 2008) (Fig.1). All FGFs are essential for embryonic development and that function postnatal as homoeostatic factors, in the response to injury, in the regulation of electrical excitability of cells and as hormones that regulate metabolism. In humans, FGFs signaling is involved in developmental, neoplastic, metabolic and neurological diseases. All FGFs act through binding extracellularly to four cell surface tyrosine-kinase FGF receptors (FGFRs 1–4) (Zhang X. et al, 2006; Krejci P. et al, 2009; Beenken A. et al, 2009). FGFRs genes have been identified in humans and mice and encode receptor tyrosine kinases (ca. 800 amino acids). These receptors contain an extracellular ligand-binding domain with three immunoglobulin domains (I, II, and III) that need to the recognition; a transmembrane domain, and a split intracellular tyrosine kinase domain. FGFRs are expressed on many different cell types and regulate key cell functions, such as proliferation, differentiation, and survival. Unlike other growth factors, FGFs act in concert with heparin or heparan sulfate proteoglycan (HSPG) to activate FGFRs and induce the pleiotropic responses that lead to a variety of cellular response. As such others FGFs, also the activity of FGF-21 is mediate by interaction with FGF receptors, but FGF-21, like others components of the same sub-family (FGF-19 and FGF-23), is not able to directly bind to the FGFRs, in fact, to interact with receptors, it needs a cofactor named β-Klotho. The cofactor β-Klotho is single-pass trans-membrane protein predominantly expressed in metabolic organs like Liver, White Adipose Tissue (WAT), Brown Adipose Tissue (BAT) and Pancreas (Ito S. et al 2000). Moreover the cofactor is important for the FGF-21 specificity of the target cells (Ogawa Y. at al 2007, Suzuki M. et al 2007). β-Klotho can interact with FGFR1, 2, 3 and 4, and FGF-21 has been reported to activate β-Klotho-FGFR1. FGFR1 is a strong candidate receptor for FGF-21 because interacts with β-Klotho more strongly than FGFR2 and 3. The FGFR1 is expressed broadly, but β-Klotho and FGF-21 expression is more restricted to metabolic tissues such as brown adipose tissue, WAT, liver, pancreas, muscle and the intestinal tract, this could explain the metabolic effects induced by FGF-21. The FGF-21–β-Klotho–FGFR complex acts by inducing a number of downstream signals such as MAP kinase phosphorylation, RAF1,

AKT1 and STAT (Ogawa Y. at al 2007,Kharintonenkov A. and Shanafelt AB, 2008). β-Klotho plays an important role in FGF-21 actions, it is required for FGF-21 effects in growth and metabolism. Studies shown that in Knock Out (KO)-β-Klotho mice were lost every effect of FGF-21action in adipose tissue. The strongly expression of β-Klotho in liver, adipose tissue and pancreas, can explain why the main effects of FGF-21 have been observed in these tissues (Fig.2) (Ito S. et al, 2000). FGF-21 has been described as an endocrine hormone due to its physiological effects on metabolism in several districts; these beneficial metabolic actions may also be associated to pharmacological effects, in diseases that affect these and others organs.

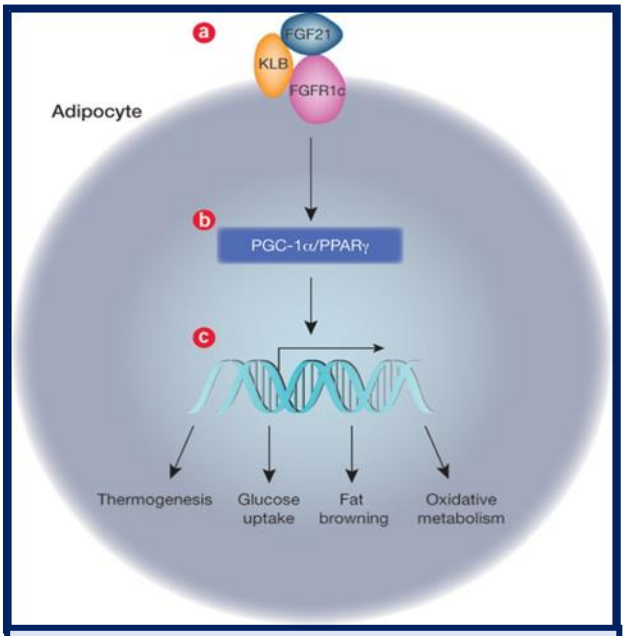


**Fig. 2-** Schematic representation of FGF-21 actions, mediate by β-Klotho co-factor in adipocytes.

## ***Physiological and Pharmaceutical actions of FGF-21***

FGF-21 is express in metabolic tissues and has several metabolic functions: it is able to regulate glucose uptake and insulin homeostasis, to influence fatty acid metabolism, to control LDLR levels and lipoprotein, to improve insulin sensitivity, and to preserve β-cell functions in diabetic animal models (Iglesias et al, 2012). FGF-21 is classified a growth factor with "hormone-like" effect, in fact together with FGF-19 and FGF-23 compounds the "FGFs endocrine subfamily".FGF-19 (the human ortholog of murine FGF-15) is expressed in the intestine and regulates bile acid synthesis in the liver in both rodents and humans (Inagaki T. et al, 2005; Lundåsen T et al, 2006). FGF-23 is produced in bone tissue and regulates phosphate and vitamin D metabolism via effects on the kidney (Fukumoto S. and Yamashita T., 2007). FGF-21 is expressed predominantly in the liver, in adipose tissue, pancreas and skeletal muscle (Muise ES. et al, 2008;Nishimura T. et al, 2000;Wente W. et al, 2006; Johnson CL. et al, 2009).The expression of FGF-21 is regulated by different transcriptional factors, such as peroxisome proliferator-activated receptor A (PPARA) in the liver (Badman MK. Et al 2007; Hondares E. et al, 2010) and proliferator-activated receptor gamma (PPARG) in adipocytes (Wang H. et al, 2008), respectively in response to stimuli like fasting, feeding, and cold. Its actions on metabolism are evident both in the fed and fasted state, but are most prominent during fasting. In this case the induction of FGF-21 in the liver is mediate by the peroxisome proliferator-activated receptor a (PPARα); it is a nuclear receptor activated by fatty acids and the fibrate class of hypolipidemic drugs (Badman et al. 2007; Inagaki et al. 2007; Lundasen et al. 2007). PPARα binds directly to the FGF-21 gene promoter to induce its transcription (Inagaki et al. 2007). In the adipose tissue the FGF-21 expression is induced by PPAR-γ and by a c-AMP-mediate pathway, which regulate the gene transcription stimulated by noradrenalin, moreover several data shown that FGF-21 expression is increased in adipose tissue of obese model mice (comparable to its expression in the liver) (Zhang, X. et al, 2008).

FGF-21 also performs metabolic functions in the pancreas, where is able to preserve β-cell function and survival by activation of extracellular signal-regulated kinase ½ and Akt signaling pathways, and inhibiting glugacon secretion, that induce a reduction of glucolypotoxicity and apoptotic effect mediated by cytokine (Wente W. et al, 2006), these

actions could correlated with a reduction of glucose levels in the blood. Moreover FGF-21 acts in the liver, where is able to induces gluconeogenesis, (increasing glucose-6-phosphate and phosphoenol-pyruvate carbokinase), it does not stimulate glycogenolisys. In a search for proteins that can modulate glucose metabolism, FGF-21 was found to increase glucose uptake into cultured 3T3-L1 mouse adipocytes and primary human adipocytes, through upregulation of glucose transporter (GLUT) 1 expression in an insulin- independent manner. Glucose uptake induced by FGF-21 is insulin-independent, probably, because FGF-21 up-regulates GLUT-1 m-RNA, and because through its binding to β-Klotho–FGFR1 complex, FGF-21 stimulates glucose uptake in differentiated adipocytes via the induction of glucose transporter-1 (GLUT1) whereas insulin acts through GLUT-4. FGF-21 needs to be express on cells for several hours, to produce a considerable response in glucose uptake, (FGF-21 effects are significantly diminished by the proteininhibitor cycloheximide), while insulin is known to work in a rapid manner, because FGF-21 activity is mediate by gene expression, unlike insulin that acts in a direct way (Kharintonenkov A. et al, 2005). To better understand the FGF-21 functions on glucose metabolism directly, in some studies recombinant FGF-21 was subsequently administered to various diabetic and metabolic disease models, all of these studies demonstrated a dramatic effect of FGF-21 in normalizing plasma glucose levels and improving insulin sensitivity. Similar results were obtained in FGF-21 transgenic animals in which levels of serum insulin were significantly decreased like at the same time glucose, cholesterol and hepatic TG levels, improved insulin sensitivity, and were resistant to diet-induced obesity. All these data can suggest a role of FGF-21 in metabolic diseases, such as diabetes. In addition to the reduction of blood glucose levels, another pharmacological effect of FGF-21 is the lowering of blood content of cholesterol and triglyceride. FGF-21 in fact is able to induces, by increasing the carboxyl ester lipase, fatty acids oxidation in the liver. Studies on FGF-21 Knock Out animal rodent model animals showed impaired Ketogenesis, hepatosteatosis and alterations in the control of glucose metabolism (Cicione et al, 2012) suggesting the crucial role of FGF-21 for these metabolic events. Some studies explain this action, suggesting that FGF-21 also induces the production of several pancreatic lipases, like pancreatic lipase, lipase-related protein and carboxyl ester lipase in the liver, in addiction it is shown that administration of FGF-21 to mice is able to stimulate the expression of some genes in adipose tissue (WAT and BAT both), mainly uncopling- protein 1 (UCP1), PPARγ co-activator 1α (PGC1α),

hormone-sensitive lipase (HSL), adipose TG lipase (ATGL) and acetyl CoA carboxylase2 (ACC2). Furthermore induction of these lipases can contribute to enhance the hepatic fatty-acids metabolism (Andrews MT. et al, 1998). Biochemical studies shown notable reductions of triglyceride and cholesterol in the liver and in the muscle of FGF-21-treatedhigh-fat diet–induced obesity (DIO) mice, accompanied by reductions of plasma AST, ALT, and ALP levels. In adipose tissue sections it was observed reduced lipid accumulation in FGF-21-treated DIO mice. The increasing of fatty acids metabolism induced by FGF-21 in the liver and other tissues like muscle and adipose tissue, is related to reduction of TG plasma levels, and reverted hepatosteatosis. In fasting response, the nuclear co-activator PPARα is induced to stimulate ketogenesis, gluconeogenesis and fatty acids metabolism, all effects are mediates by the action of FGF-21. It is shown that FGF-21 also regulates fasting promoting lipolysis in WAT from murine adipocytes and ketogenesis, and in liver in response to fasting directly induced by PPARα although this FGF21-induced lipolysis; these evidences demonstrate a role for the PPARα-FGF-21 endocrine signaling pathway, in regulating several metabolic and behavioral aspects of the adaptive response to starvation (Kurosu and Kuro, 2009). The favorable effect on hepatic steatosis might be related also to the inhibition of the maturation of sterol regulatory element-binding protein-1c, a transcription nuclear factor that activates all genes required for lipogenesis.

It is shown that FGF-21 is also involved in the regulation of the body-weight and body fat mass. It is observed elevated blood concentrations of FGF-21 in obesity; some studies demonstrate a weight loss in diabetic no human primates, after chronic administration of FGF-21 (Kharitonenkov A, et al 2007), confirming that in obesity there is a FGF-21-resistant state; to accept this in FGF-21 overexpressing mice it is evident a reduced fat mass content and reduction of resistance to obesity (Ficher ML. et al, 2010). Moreover FGF-21 reduces physical activity and promotes torpor in mice to save energy by reducing physical activity and body temperature energy.

Recently, many studies have investigated the pharmacological effects of FGF-21 in humans. Due to its involvement on the reduction of glucose levels in the blood, FGF-21 can be considered such as diagnostic marker to identify diabetes mellitus II in humans. Several authors related increased plasma levels of FGF-21 with an alterated glucose metabolism; elevated circulating concentrations of FGF-21 have been observed in insulin

resistant diabetes (Type II diabetes), whereas FGF-21 plasma levels are decreased in autoimmune condition (Type I diabetes) (Xiao Y. et al 2011). As demonstrate in animal models, FGF-21 plays a role also in obesity in humans: increased FGF-21 serum levels have been found to be associated with obesity in both children and adults, indicating a connection between FGF-21 and body fat mass (Reinehr T et al 2012). It seems that FGF-21 can have also a role in cardiologic and renal disease. In fact, it is recently reported that FGF-21 levels may be altered in coronary and renal diseases. In a clinic study, median plasma FGF21 levels were significantly higher in CHD patients than in control subjects. Moreover in CHD patients with diabetes or hypertension, or both FGF-21 levels were higher than those of patients without these comorbidities. In this study it is observed that plasma FGF-21 concentrations are positively related with TG, fasting blood glucose, apolipoproteinB100, insulin, and HOMA index of insulin resistance, but negatively with HDL and apolipoprotein A1. These evidences can suggest a positive relationship between FGF-21 and adverse lipid profile in CHD patients and a possible compensatory response or resistance to FGF-21 (Lin Z et al 2010). Others studies have been demonstrated that circulating FGF-21 levels are also associated with renal function and progressively increased from early- to end-stage renal disease (ESRD). It is observed FGF-21 serum levels to bew8–15 times higher in long-term dialysis patients compared with healthy subjects. FGF-21 mighty has a role in insulin resistance in ESRD patients. These data shown that FGF-21 is positively correlated with inflammatory markers such as interleukin-6, fibrinogen, high-sensitivity C-reactive protein and HOMA-IR and negatively with residual renal function in a group of 72 non-diabetic peritoneal dialysis patients. Increased FGF-21 plasma concentration in dialysis patients, could be due to impaired renal excretion combined with compensatory mechanisms to counteract metabolic stress and/or insulin resistance and FGF-21 resistance in peripheral tissues (Han SH et al 2010).

Finally a recent study shown that FGF-21 expression may be induced during multiple intracellular stress signals, such as oxidative stress, amino acid deficiency and also unfolding protein response (UPR). In UPR FGF-21 is induced by ATF4 and its pathway (PERK/eIF2-α/ATF4), that is strongly expressed in UPR (Schaap et al 2013). Several studies highlight FGF-21 also like a marker of mitochondrial disease in different kind of disorders (Gavrilova R and Horvath R 2013). Mitochondrial dysfunction can result in central nervous system damage, especially in the brain stem and cerebellar

region. Increased plasma FGF-21 levels has been found in spinocerebellar atrophy (SCA) patients (Su et al 2012); this can suggest a role of FGF-21 in central nervous system, despite the expression and functions of FGF-21 in the brain are so far unknown. All these data underscore the multiplicity of physiological and pharmacological actions of FGF-21 in various tissues. All the effects of FGF-21 are mediated by the interaction with its receptors, which by forming a protein complex with the coactivator β-Klotho, can activate a series of downstream effects, including the induction of expression of certain proteins which in turn activate a series of responses to various environmental stimuli. As largely demonstrate, one of the factors involved in mainly activity of FGF-21 is the transcriptional co- activator PGC-1α whose expression is strongly increased by FGF-21, in different tissues.

## ***FGF-21 induces PGC-1α***

FGF-21 can play its metabolism regulation functions by inducing the production of proteins able to activate a number of downstream pathways; in some cases FGF-21 cannot perform their functions without the involvement of other factors, such as PGC-1α.In several studies it is observed that administration induces, in mice liver, the expression of the transcriptional co-activator *peroxisome proliferator-activated receptor c protein-1α,* which interacts with several different DNA-binding proteins to regulate oxidative metabolism in response to several physiologic stimuli. In the liver, the induction of PGC-1α can stimulates the transcription of genes involved in gluconeogenesis, fatty acid oxidation, and ketogenesis. Importantly, FGF-21 is unable to induce gluconeogenic gene expression in transgenic mice lacking PGC-1α. Moreover, mice lacking FGF-21 cannot induce PGC-1α expression in response to a prolonged fast and have impaired gluconeogenesis and ketogenesis (Potthoff MJ et al 2009). Thus, others metabolic actions of FGF-21 are mediated in part through PGC-1α. In a recent study it is reported the ability of FGF-21 in the endogenous cold response. FGF-21 expression is also necessary for the cold-induced recruitment of brown-like adipocytes in WAT; in fact FGF-21 administration can stimulate thermogenic gene response in both BAT and thermogenically competent WAT depots. This action is related with a massive induction

of PGC-1α protein, because it is required for the thermogenic effects (Ffolliott M. et al 2012). Today FGF-21 is the only secreted protein known induced by cold that can increase, by inducing PGC-1α expression, the appearance of brown-like/brite adipocytes in WAT depots. In adipose tissue PGC-1α acts in concert with both AMPK and Sirtuin-1 (SIRT-1) to regulate energy homeostasis in response to environmental and nutritional stimuli; available data shown that treatment with FGF-21 can increase AMPK and SIRT-1 activities via LKB1 in human adipocytes. SIRT-1 activation is mediated by the alteration of $NAD^+$ levels, resulting after FGF-21 treatment. The activation of these metabolic regulators results in enhancement of mitochondrial oxidative function, clarifying the FGF-21 beneficial effects on metabolism and correlation between FGF-21 mediate PGC-1α effects (Chau et al 2010). Together these findings suggest the crucial role of PGC-1α in FGF-21 main functions, band assumes a correlation between the two molecules also in other districts.

## ***Peroxisome proliferator-activated receptor c co-activator protein-1α (PGC-1α)***

Transcriptional co-activators are proteins able to regulate gene expression without recognizing and binding specific DNA sequences, but identifying and binding proteins, transcriptional factors, that requiring co-activators or co-repressors to act. PGC-1α was identified in a differentiated brown fat cell line, it belongs to a family of co-activators with PGC-1β and PGC-1 related co-activator (PRC), that have functions in mitochondrial physiology. PGC-1α and PGC-1β contain a conserved amino acids sequence, LXXL, which represents the domain able to interact with nuclear factors and host cell factor 1 interacting motif (Lin et al 2003); PRC also contains an activation domain and an RNA-binding domain, but the homology with both other members of the family is so limited. PGC-1α gene is located on chromosome 5 in mice (chromosome 4 in human); it is a 797 amino acids long protein (798 aa in human). PGC-1α powerfully regulates oxidative and mitochondrial metabolism in a number of tissues, induces mitochondrial biogenesis, it is able to interact directly with the basal transcriptional machinery, and the mediator complex, to activate the transcription. The co-activator can induce gene expression also

recruiting chromatin-modifying enzymes, such as the histone acetylase p300, to open the chromatin for the activity of the transcription complex. In addition, PGC-1α can regulate gene expression by coordinating post-transcriptional events, because interacts with the splicing machinery. Structurally, the PGC-1α protein has 200 amino acids at N-terminus in which resides the transcriptional activation domain, that interacts with SRC-1, CBP/p-300, steroid receptor co-activator 1, c-AMP and several histone acetyl-tranferase complexes. In the central region (aa 200-400) there is the repression domain, that binds some transcription factors. The C-terminal domain contains a region which recognizes and binds proteins involved in RNA processing, like hnRNP proteins (Krecic and Swanson, 1999) (Fig.3). Specific post-translational modifications such as phosphorilation, acetylation, and methylation on specific amino acids residues modulate the activity of PGC-1α as well as interactions with other proteins. Sirtuin-1 (SIRT-1) is an important regulator of PGC-1α activity, it acts through lysine de-acetylation; moreover SIRT-1 can increase the rate of transcription maintaining the de-acetylated active form of the protein (Gerhart-Hines et al 2007).

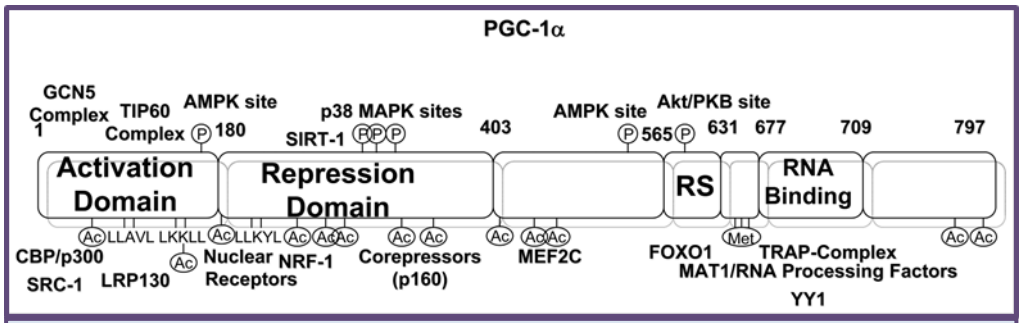


**Fig. 3**- Architecture of the PGC-1α transcriptional co-activator. PGC-1α contains several functional domains that correlate with the interactions with different proteins and complexes.

PGC-1α is expressed in tissues which have high energy demand, like heart, skeletal muscle, liver, brain, kidney, brown adipose tissue (BAT) (fig.4). In muscle and BAT,

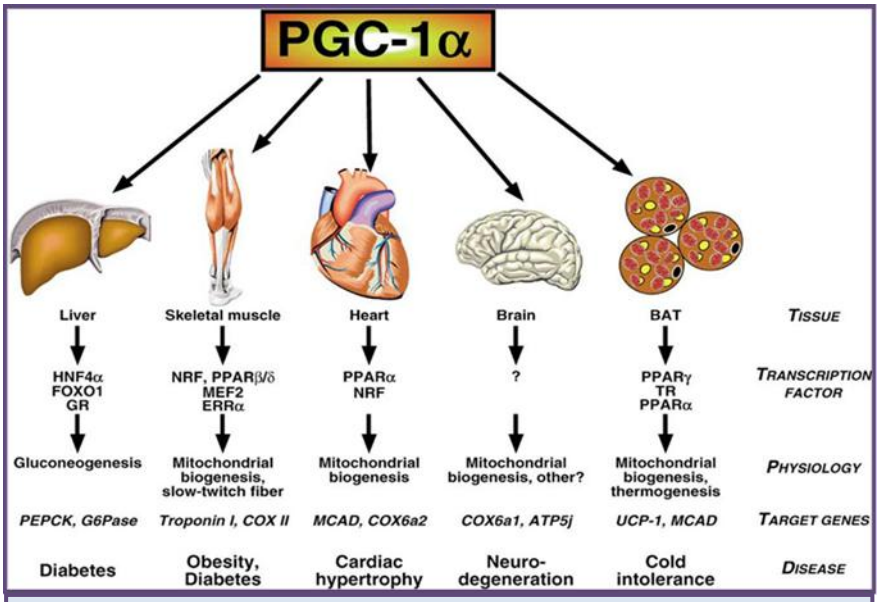


**Fig. 4**- Representation of expression and functions of PGC-1α in different tissues.

PGC-1α induces nuclear and mitochondrial genes to stimulate mitochondrial biogenesis, this effect is accompanied with the co-activation of nuclear respiratory factors 1 and 2 (NRF1, NRF2) and the estrogen-related receptor a (ERR-a) (Mootha et al 2004); NRF1 and NRF2 enhance the expression of mitochondrial transcription factor A (mt-TFA) that translocate in the mitochondrial matrix, and stimulate mt-DNA replication and gene transcription. Moreover NRF1 and NRF2 induce expression of members of the electron chain complex (cytochrome C, ATP synthase, and CoXIV) (Scarpulla, 2002). PGC-1α exerts its functions also in Central Nervous System (CNS) which is widely known that is the most vulnerable tissue to oxidative stress. It is shown that an impaired PGC-1α expression in brain in concert with mitochondrial dysfunctions, can contribute to neurodegeneration in most susceptibles neural cells (Weydt et al 2006) so, PGC-1α has a notable task in normal brain functions and a pivotal role in the oxidative stress response, because it regulates the expression of important factors against ROS, such as copper/zinc SOD1, manganese SOD1, and glutathione peroxidase 1 (St Pierre et al 2006). Some evidences indicate that in mice, PGC-1α deficiency can cause behavioral changes like anxiety and hyperactivity as well as hind limb clasping. These changes can be associated with spongiform-like vacuolization primarily in the striatum associated with

gliosis and leads to reduced expression of several brain-specific genes that are all associated with normal brain functions. Some data suggest also an important role of PGC-1α in response to neurotoxins in neuronal maintenance in Substantia Nigra (SN) and Hippocampus (CA1) in neurons, that are more susceptible to oxidative stress and neurodegeneration (Lin et al. 2004). PGC-1α also seems to be involved in the control of neurite growth and neuronal synaptic function (Cowell et al. 2009). Some studies demonstrate a role of PGC-1α in the onset and progression of neurodegenerative diseases. In postmortem brain samples of patients with Hungtington's Disease (HD), it was found a decreased level of PGC-1α mRNA (Cui et al. 2006;Weydt et al. 2006). A mutant form of the Huntington protein, which leads to mitochondrial dysfunction and neurodegeneration, represses PGC-1α. Loss of PGC-1α in HD mice aggravated neurodegeneration and its over-expression can rescue cells from the deleterious effect of HD (Cui et al. 2006). Moreover, PGC-1α KO mice show HD like phenotype and neuronal lesions suggesting that PGC-1α is crucial for maintenance of striatal function. Additionally, PGC-1α SNPs are associated with the age of onset of HD. Polymorphisms of PGC-1α are also associated with the onset of Alzheimer's Disease (AD) (Qin et al. 2009). Furthermore in a Parkinson's Disease (PD) mouse model with deficiency of PGC-1α, degeneration of dopaminergic neurons the SN was increased and associated with oxidative damage (St-Pierre et al. 2006;Bender et al. 2006).

There is a significant evidence that mitochondrial dysfunction and oxidative stress are a causative or at least ancillary factor in the pathogenesis of major neurodegenerative diseases. The loss of regulation of inflammatory markers induced by oxidative stress blocks the antioxidant systems, and causes inability to synthesize new proteins, disorders of the proteasome, accumulation of misfolded proteins and, the conformational change in key receptors involved in metabolism and cell signaling (Sevcsik et al. 2011). All these factors, accumulated slowly over time contribute to the establishment of neurodegenerative disorders that may manifest as abnormalities that cause the state of chronic oxidative (Halliwell 2006). Parkinson's Disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (ALS) are examples of neurodegenerative diseases in which there is an important involvement of oxidative stress, as well as in cases of stroke, trauma, and seizures (Beal et al. 2000; Ienco et al. 2011); moreover, oxidative stress is a common pathogenic mechanism underlying many major psychiatric disorders (Wang et al. 2009). It is shown that activators of PGC-1α such

as others molecules that can induce it expression, like resveratrol (RSV), have a neuroprotective effect in acute and chronic brain injury as well as in neurodegenerative diseases suggesting also a role for PGC-1α in modulating the outcome of the disease (Sun et al. 2010). Due to its action on regulation of cell metabolism, mitochondrial biogenesis, and regulation of antioxidant enzymes, a number of evidences demonstrate an important involvement of PGC-1α in neurodegenerative diseases. Studies shown role for PGC-1α in modulating the outcome of the disease, brain injury, and indicate PGC-1α and SIRT-1 like potential targets to treat neurodegenerative diseases such as Parkinson's Disease, and more recently, studies shown that PGC-1α can also have a neuroprotective effect in dopaminergic neurons in animal model of Parkinson's disease.

## ***Parkinson's Disease and neuroprotective effects of PGC-1α in dopaminergic neurons***

Parkinson's disease represents one of the strongest cases based in part on post-mortem studies that have shown mitochondrial impairment (e.g. reduced complex I activity) and oxidative damage in idiopathic PD brains. Clinically it is manifested by the tetrad of motor manifestations of tremor, rigidity, slowness of voluntary movements, and poor balance (Fahn, 2003). PD seems to be essentially as a sporadic condition, that is, in absence of any evidence of genetic linkage, but, in a handful of cases, can derive from a variety of genetic defects, such as mutations in α-synuclein (Polymeropoulos et al. 1997), parkin, DJ1 (Bonifati et al. 2003), PINK1 (Valente et al. 2004), and LRRK2 (Zimprich et al. 2004) it is inherited (Dauer and Przedborski 2003). These different forms have not distinguishable symptomatology, and present the same biochemical alterations: a drastic deficiency in brain dopamine. The reason for this reduction in brain dopamine is because all of the ascending dopaminergic pathways in the CNS degenerate, albeit to variable degrees. For instance, among these pathways, the nigrostriatal pathway-which is composed of dopaminergic neurons residing in the ventral midbrain nucleus, the SN, and which projects upward to the striatum, is consistently the most severely affected. It is remarkably, however, that PD neurodegeneration not involves only the dopaminergic systems, but the widespread neuronal loss can be detected in other catecholaminergic and noncatecholaminergic nuclei in fact motor symptomatology is due to neurodegeneration

in the dopaminergic systems, whereas others manifestations of PD, such as pain, cognitive impairments, and constipation, are more likely linked to neurodegeneration in different circuits. Post mortem samples of PD patients present the "Lewy bodies", that are intraneuronalproteinaceus inclusions, of insoluble proteins, overall α-synuclein found within many of the spared neurons in nearly all affected brain areas (Shults, 2006) and gliosis (Przedborski, 2007). Several studies showed that the initial degeneration of dopaminergic neurons in PD is increased by production of ROS originated in all cell compartments. The elevated ratio of ROS can damage all cell systems and molecules. The primary source of production of ROS is precisely the metabolism of dopamine, neurotransmitter that resides in synaptic vesicles, but that can diffuses into the cytosol when it is produced in excessive amounts, because its metabolism is by its oxidation performed by the enzymes Monoamino Oxidase (MAO), which mainly produces $H_2O_2$ and dihydroxyphenylacetic acid (DOPAC) (non-enzymatical and spontaneous auto-oxidation of dopamine produces $O_2$- and reactive quinones). Enzymatic oxidation of dopamine to $H_2O_2$ causes also increased formation of oxidized glutathione, suggesting the occurrence of oxidative stress and impairment of a major antioxidant system; $H_2O_2$ in dopaminergic neurons interact by SOD with nitric oxide radicals (NO) to consequently generate peroxynitrite, moreover the abundance of metals in these neurons cause the production of radical OH. It is known that in PD, another cause of ROS and oxidative stress resides in mitochondrial alterations. Furthermore, NO accumulation can form adducts with mitochondrial enzymes and dysregulate mitochondrial function (Ebadi and Sharma, 2003; Yamamoto et al., 2007). Keeney at al observed a central role of mitochondrial dysfunction in PD, due to deficits in the subunits and activity of mitochondrial complex I (NADH ubiquinone Oxidoreductase) of the electron transport chain and a decrease in the function of Complex III, found in blood platelets, lymphocytes and SNc of PD patients. Moreover several data confirm the predominant role of mitochondrial disfunction on PD. It was shown that SNc dopaminergic neurons have high amount of mt-DNA deletions in PD patients, if compared with other neuronal populations in brain- and age-matched controls (Bender et al. 2006). A related study identified that nigral neurons from PD patients contain high levels of clonally expanded somatic mt-DNA deletions leading to mitochondrial dysfunction (Kraytsberg et al. 2006). Mitochondrial DNA is particularly susceptible to oxidative damage. The lack of the physical protection histones, vicinity with ROS source (electron transport chain) and the

absence of inefficient repair mitochondrial machinery can explain this susceptibility. Recent studies suggest that also Endoplasmatic Reticulum stress by accumulation of unfolded and/or misfolded proteins contribute to PD (Wang and Takahashi 2007). Other evidence demonstrate that inflammation can also contribute to ROS production and has been implicated in the pathogenesis of PD; in fact, activation of microglia in response to injury is associated with an up-regulation of inducible nitric oxide synthase (NOS) resulting in increased production of NO (Drechsel and Patel 2008). Finally it is also observed a contribute to PD pathogenesis by Endoplasmatic Reticulum stress by accumulation of unfolded and/or misfolded proteins (Wang and Takahashi 2007). All these evidence focused the importance of mitochondrial functions in the pathogenesis of neurodegenerative diseases of PD. As largely shown one of the crucial molecules for the mitochondrial activity is PGC-1α, it, in fact, plays a pivotal role in the expression of genes responsible for the detoxification of ROS, including copper/zinc SOD1, manganese SOD2, and glutathione peroxidase 1 (St-Pierre et al. 2006). Interestingly, SIRT-1, activator of PGC-1α, plays a similar role in oxidative stress function in combination with Fox-O transcription factors (Brunet et al. 2004). For this reason, PGC-1α plays an important role in normal brain function and a major role in the oxidative stress response. These evidences have suggested a role of PGC-1α on protection against oxidative stress in neurodegenerative disease. Mudò et al, have recently studied and demonstrate that in transgenic overexpressing mouse model PGC-1α is able to confer neuroprotection to the dopaminergic neurons in SN, on MPTP model of Parkinson's disease. In fact it was observed, in the SN of overexpressing PGC-1α mice the number of neurons, on MPTP treated mice were essentially unchanged to controls and wild type ones. This study showed the PGC-1α transgenic mice were significantly more resistant to MPTP-induced neurotoxicity than the wild-type mice as reflected also by smaller reduction of dopamine and DOPAC. In addition, the increase of dopamine/DOPAC ratio was lower in the PGC-1α transgenic mice as compared to controls. Moreover in overexpressing PGC-1α model it was evident an increased rate of respiratory control rate in mitochondria as compared to organelles from wild-type mice, indicating that the neuronal capacity for ATP production is higher in these mice compared to wild-type animals. Moreover the authors observed that resveratrol (RSV) treatment in murine dopaminergic cell line (SN4741), is able to increase the levels of SIRT-1 and activated PGC-1α, by reducing acetylation of the

protein. RSV increases the levels of PGC-1α in the dopaminergic cells by affecting gene expression.

These data, together, underscore the importance of PGC-1α signaling in neuroprotection of dopaminergic neurons, and adds to previous studies on the role of PGC-1α in models of neuronal excitotoxicity and cell stress (St-Pierre et al. 2006; Lu et al. 2010).It is largely known that PGC-1α expression is enhanced in several metabolic tissues by growth factors as FGF-21, knowledge on the involvement of FGF-21 in mitochondrial disorders associated with important antioxidant activity of PGC-1a we suggest a possible correlation between these molecules at the level of the central nervous system.

# ***Aims***

FGF-21 is a growth factor that plays a role in balance energy excerting hormone-like effects functions on metabolism. It has metabolic actions and is involved in a protection mechanism against oxidative stress in different tissues, such as liver, muscle and adipose tissue. Moreover FGF-21 is able to induce PGC-1α expression, to exert its functions. PGC-1α is a transcriptional co-activator with important neuroprotective effects in dopaminergic neurons. On the basis of these evidences the aim of the present study was to investigate if FGF-21 induces PGC-1α in human dopaminergic neurons. To this end we treated human dopaminergic neurons cell line with FGF-21, we studied its effect on PGC-1α gene expression, protein activity, including its mitochondrial effects. Another aim of this work was to explore the possible expression and functions of FGF-21 in the brain; we performed these studies, in animal model of oxidative stress induced with kainic acid treatment.

# Materials and Methods

## Cell culture

We performed our in vitro experiments using human mesencephalon neuronal precursor cells (MESC2.10 cells), cultured in in poly-Dlysin (Sigma, St Louis, MO, USA) coated flasks (75cm2) in Dulbeccos modified Eagle medium (DMEM)/F12 medium (Gibco, Invitrogen, Calrsbad, CA, USA) supplemented with B27 (Gibco) and Penicillin/streptomycin and human basic FGF 20ng/ml (Peprotech, Rocky Hill, NJ, USA). Cultivating cells for 6 days on poly-Dlysine/ laminin (Sigma) coated wells at a density of 30000 cells /cm2 in a medium containing 1µg/ml tetracyclin (Sigma), we were able to induce the differentiation into dopaminergic neurons (Lotharius et al., 2002; Di Liberto et al., 2012), the medium was changed every second day. To verify the state of differentiation we monitored the expression of markers for dopaminergic neurons such as tyrosine hydroxylase and dopamine transporter. Microglial and astrocyte cells were prepared from newborn of rodent brains, and cultured fetal calf serum in DMEM for two weeks (Lindholm et al., 1992; Mäkelä et al., 2010). The human hepatocyte cell line Huh7 was used as a positive control for FGF21 expression (Do et al., 2012).

## Animals

In vivo experiments were performed using three months old female Wistar rats. Animals were kept under environmentally controlled condition, ambient temperature 24 °C, humidity 40 %, and 12 h light/dark cycle with food and water ad libitum. Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national (D. L. n. 116, G. U., suppl. 40, 18 February 1992) and international laws and policies (EEC Council Directive

86/609, OJ L 358, 1, 1 December 12, 1987). Rats were injected with KA (0.35 µg per side) in lateral ventricle, (ICV) and killed at different time-points after 24, 48 and 72 hours; controls received same treatment injection with saline. Animals were killed under deep anesthesia followed by cervical dislocation. Collected brains were immediately frozen in isopentane and stored at -80°C.

## ***Immunoblotting***

We treated the MESC2.10 differentiate cells with 50 ng/ml of FGF-21 (R&D Systems, Minneapolis, MN, USA). After 24h cell samples were lysed with RIPA buffer containing 150mM NaCl, 1% Triton-X 100, 0,5% sodium deoxycholate, 50mM, pH 8, and 0, 1% sodium dodecyl sulfate (SDS). Using SDS-PAGE, 30–30 µg of protein was separated, transferred to nitrocellulose membranes (Hybond-C-Extra, Amersham Biosciences, Buckinghamshire, UK), blocked for 1 hour at room temperature in TBST and 5% skim milk, and incubated overnight at 4°C with primary antibodies. We used anti-α-PBEF/NAMPT (1:1000; Abcam, Cambridge, UK), anti-SOD2 (1:5000; AbFrontier, Seoul, Korea), anti-Trx2 (1:1000; AbFrontier), anti-PGC-1α (1:5000; Calbiochem, San Diego, CA, USA), anti-COX IV (1:2000; Abcam), anti-mtTFA (1:1000; Abcam), anti-FGF21 (1:3000; Novus Biologicals, Littleton, CO, USA) and anti-β-actin (1:5000; Sigma). The membranes were washed in TBS 5%, Tween 20 buffer and incubate for 1h at room temperature, with peroxidase-conjugated secondary antibodies: (1:2500; Jackson Immuno Research, West Grove, PA, USA) were. Super Signal West Pico chemiluminescent substrate (Thermo, Waltham, MA, USA) was used for visualization of the bands, and ImageJ software for their quantification (http://rsb.info.nih.gov/ij/).

Different brain regions of adult mouse were dissected under stereomicroscopy and frozen in cooled isopentane. Brain tissue pieces were homogenized in cold buffer containing 50 mMTrisHCl pH 7.5, 100 mMNaCl, 1 mM DTT, 5% NP-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.1 mMphenylmethylsulfonyl fluoride, 100 µM sodium orthovanadate (Na3VO4) and 1 mM EDTA (Sigma-Aldrich, St. Louis, MO, USA). The homogenate was left on ice for 30 min and centrifuged at 10,000 × g for 15 min at 4°C to yield supernatant fractions that were stored at -80°C until use. To obtain enough material, the two SN were pooled. 30–40 µg of protein was subjected to immunoblotting as above

using primary antibodies anti-FGF21 (1:3000) and β-actin as control. The membrane was processed as above using secondary antibodies and visualization of bands was made by enhanced chemiluminescence.

## ***Immunohistochemestry***

To perform Immunohistochemestry experiments brains were taken from adult rats and mounted into Tissue-Tek (Sakura Finetek, Netherlands) and frozen on dry ice 14μm thick coronal sections were prepared with a cryostat (Micron, HM 500 M, Walldorf, Germany) as described (Putkonen et al., 2011; Koivuniemi et al., 2013). Sections were gradually brought to room temperature and dried, than fixed in Paraphormaldeyde 4% pH 7.4 for 15 minutes at room temperature, after a pre-incubation in blocking solution for 30 minutes (phosphate-buffered saline ⁄ 5% bovine serum albumin ⁄ 0.1% Triton-X-100) at 4°C, sections were incubate overnight at 4°C in humid chamber with primary antibodies (anti FGF-21 Novus Biologicals NBP1-59291 diluition 1:2000; anti TH Covance MMS-503P diluition 1:1000; anti-GFAP Chemicon MAB360 diluition 1:500; anti CD11 BD pharm 550299 diluition 1:50; anti CNP-ase Sigma C5922 diluition 1:500).

Incubation with primary antibodies was followed by secondary antibodies Alexa 488 or 594-conjugates. The nuclei were counterstained with bisbenzimide⁄ Hoechst (Sigma33258).

Sections were analyzed at r.t. with a confocalmicroscope (LSM 510 Meta; Carl Zeiss, Inc. in Minerva Foundation Insitute, Biomedicum, Helsinki) using ISM aim software and a magnification 40X.

## ***Immunoprecipitation***

To made immunoprecipitation experiments we used lysates from human dopaminergic cells treated with 50ng/ml FGF21 for 24 h alone or together with 10mM nicotinamide (Acros organics, Geel, Belgium). PGC-1α antibody (Calbiochem) at the dose of 1,5 was added to 500µg of cell lysates overnight at +4°C under constant rotation. 50µl of Protein G-agarose (Roche, Basel, Switzerland) was then added to the samples and incubated for 6 hours. After the incubation samples were recovered by centrifugation., beads were washed three times with RIPA buffer and lysates were run on 8% SDS-PAGE, followed by transfer and using either anti-PGC-1α antibodies (1:1,000; Cell Signaling) or anti-acetylated-lysine antibodies (1:1,000; Cell Signaling). The intensity of the bands reveals the degree of acetylation of PGC-1a under different conditions as described above using first anti-acetylated lysine antibodies and then anti-PGC-1α antibodies. The quantifications of the bands were done using ImageJ software. The degree of acetylation of PGC-1α is an index for the activity of this protein in the cell (Rodgers et al, 2005; Mudò et al., 2012).

## ***Luciferase Assay***

To analyze gene expression levels of PGC-1α in dopaminergic neurons we made a luciferase assay. The neuronal precursors were transfected with 0,5µg of the PGC-1α–luciferase reporter constructs or the control pGL3 promoter constructs together with 0,025µg RenillapRL-TK plasmid using Fugene (Promega, Madison, WI, USA). Cells were incubated for 2 days in the differentiation medium and 50ng/ml FGF21 was added for an additional 24h. To control for transfection efficiency, 0.02 µg Renilla luciferase pRL-TK was used. Cells were harvested after 48 h using Passive Lysis Buffer, and the Renilla and the firefly luciferase activities were measured using a luminometer (Promega, Biofellows, Helsinki, Finland) (Kairisalo et al. 2009). Results are shown as fold increase in luciferase normalized to Renilla activity.

## ***NAD+/NADH levels***

To estimate levels NAD+ we used the NAD+/NADH assay kit (Abcam) according to manufacturer's protocol. The absorbance was measured at 450nm was using Multiscan MS Version 3.0 spectrophotometer.

## ***Real-time analyses of mitochondrial respiratory capacity in human dopaminergic neurons***

Differentiated for 5 days cells, were treated with 50ng/ml FGF2. After a 24h-stimulation, cells were plated on Seahorse 96well plate (Seahorse Bioscience, Boston, MA USA). Medium was changed to HCO3-free DMEM (Sigma) containing 10mM pyruvate, 1mM l-glutamine and 10mM glucose 1h prior to analyses and keeping cells at +37°C without CO2. Relative oxygen consumption in dopaminergic neurons was determined in real-time using the Seahorse XFe96 analyzer (Seahorse Bioscience) by determining oxygen consumption as response to the addition of various chemicals. Three min cycles were run for every measurement and the following compounds were subsequently added using the Mitostress kit (Seahorse): 1µM Oligomycin (ATP synthase inhibitor), 0,8µM Carbonyl cyanide 4- (trifluoromethoxy) phenylhydrazone (FCCP) (mitochondrial uncoupler), 1µM Rotenone (complex I inhibitor of the respiratory chain) and Antimycin A (complex III inhibitor).

## ***Mitochondrial DNA copy number***

We isolated DNA from human dopaminergic neurons using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's protocol. To measure relative mitochondrial DNA (mtDNA) copy number we used quantitative PCR and

compared the ratio of mtDNA to nuclear DNA and the method described previously (Xieetal., 2013). In brief, the ND1gene was used for quantifying mt-DNA and human globulin (HGB) gene for nuclear DNA. The primer sequences used were: forward primer ND1-F: 5´-CCT AAA ACC CGC CAC ATC T-3´, reverse primer ND1-R: 5´-GAG CGA TGG TGA GAG CTA AGG T-3´; forward primer HGB-F: 5´-GTG CAC CTG ACT CCT GAG GAG A-3´, and reverse primer HGB-R: 5´-CCT TGA TAC CAA CCT GCC CAG-3´. The PCR reaction mixture contained 5ng DNA and 200nmol/l of forward and reverse primers in 1X SYBR Green Mastermix (Roche). The reaction was carried out at 95°C for 10min followed by at 95°C for 15s and 60°C for 1min using 40 cycles. Each sample was run in triplicates on a 96- well plate and water was used as negative control. Quantitative-PCR amplification was performed using Light Cycler 480 II instrument (Roche) as described (Do et al., 2012; Hyrskyluoto et al., 2013). The ratio of mt-DNA to nuclear DNA reflects the concentration of mtDNA per dopaminergic cell.

### ***Electron microscopy and mitochondrial density analysis***

Human dopaminergic neurons stimulated with 50ng/ml FGF21 for 24h were fixed with 2,5% glutaraldehyde in PBS for 1h at room temperature and washed two times with H2O for 1hour. Postfixation was done using 1% osmiumtetroxide. The embedding and sectioning procedures were done essentially as described before (Korhonen et al., 2008). The sections were stained with lead citrate and uranyl acetate and viewed using a Jeol JEM-1400 transmission electron microscope (Jeol Ltd., Tokyo, Japan) equipped with GatanOrius SC 1000B bottom mounted CCD-camera (Gatan Inc., USA). Mitochondria identified in the EM pictures of the cells were marked manually and the relative surface area was calculated using the ImageJ software. Results were pooled from different sections of control and FGF21-treated cells and the density of mitochondria was compared.

## ***Animal experiments***

To activate microglial cells rats were injected with kainic acid (Sigma, Helsinki, Finland) into the lateral ventricle (0.35 μg/μl) in a volume of 0.5 μl per side as described previously (Sokka et al., 2007; Putkonen et al., 2011). Animals were kept for different times: 24 hours, 48 hours and 72 hours and decapitated in deep anesthesia, and brains were rapidly dissected, frozen in isopentane, cooled in liquid nitrogen and stored at −70 °C until being processed for immunohistochemistry as described above.

Experiments were approved by the ethical committee and carried out in accordance with the European Communities Council Directive (86/609/EEC). Every attempt was made to reduce the number and to minimize pain and suffering of animals.

# Results

## *FGF-21 is able to increase levels and deacetylation of PGC-1α in cultured human dopaminergic neurons*

The first aim of the present work was to analyze the effects of FGF-21 on PCG-1α expression and activity in human dopaminergic neurons. To carry out our research we cultivated midbrain progenitor (MESC2.10) cells from human embryonic brain that were further differentiated into dopaminergic neurons in culture (Lotharius et al., 2002). We treated the cell line with 50ng/ml FGF-21 for 24hours. After 24 hours we used cell lysate to perform immunoblotting experiments employing an anti PGC-1α antibody. We observed that the FGF-21 treatment led to an increase in PGC-1α protein as shown in the Figure 5. To clarify whether this involved increased gene expression we used the PGC-1α-promoter linked to a luciferase reporter gene, transfecting our neuronal precursor cell line with an appropriate plasmid construct. Our results indicate that the gene activity of the PGC-1α promoter was increased by FGF-21 treatment with no changes in the activity of the pGL3 basic-promoter used as control (Fig. 6).

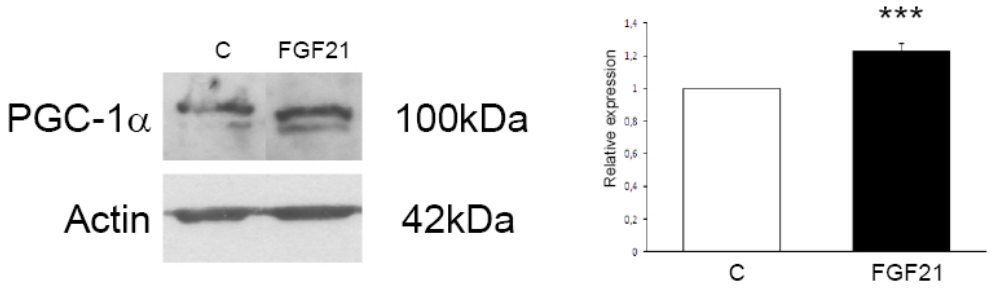


**Fig. 5- FGF-21 increases PGC-1α levels in dopaminergic neurons**.- Immunoblots. β-actin was used as a control. The levels of PGC-1α were increased by FGF21 as compared to control. Left, typical immunoblot. Right, quantification done using ImageJ software. Values are means± SD, n=4, ***p<0.001 for FGF21 vs controls.

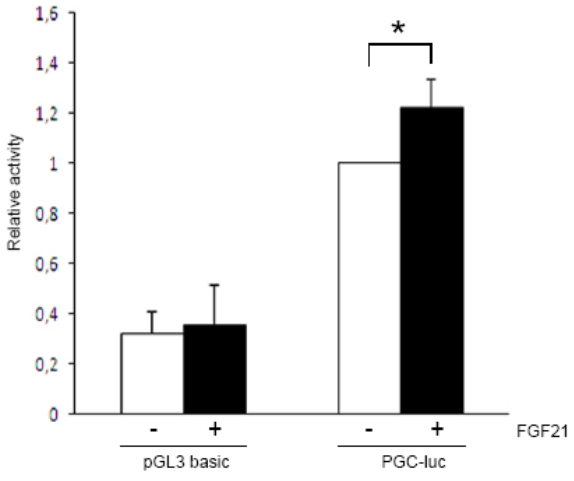


**Fig.6: FGF-21 enhances the activity of PGC-1α gene promoter-** Gene promoter assays. Cells were transfected with the pGL3 basic plasmid and the PGC-1α promoter plasmids linked to a luciferase reporter gene. Cells were stimulated with FGF21 for 24h and the luciferase activity was measured and corrected for that of Renilla as described as described in Methods. FGF21 enhanced PGC-1α gene activity but not that of the control pGL3 promoter. Values are means± SD, n=4, *p<0.05 for FGF21 vs controls.

We also observed an increase in PGC-1α mRNA levels in FGF21-treated dopaminergic neurons using quantitative PCR (data not shown). These data indicate that FGF-21 treatment is involved in the increase the PGC-1α of protein levels, and genic expression. The activation of functions of PGC-1α depends by its post-translational modifications, such as the removal of acetyl groups in specific amino acidic residues. It is know that the de-acetylation of the lysine can activate the gene co-activator function of PGC-1α, in fact this kind of modification facilitates the binding with other proteins that recognize and bind DNA.

To study whether FGF-21 could activate PGC-1α protein we performed immunoprecipitation to evaluate the degree of acetylation of specific lysine residues (that need to activate the protein). Experiments followed by immunoblotting using anti-PGC-1α and anti-acetylated lysine antibodies.

In the treated cell samples with FGF-21 was evident a reduction of the relative degree of acetylation of PGC-1α as compared to control (Fig.7). Collectively these data indicate that FGF-21 is able to increase the level of protein in the cell, and can increase also the activation status of PGC-1α in the human dopaminergic neurons.


**Fig.7- FGF-21 decreases the deacetylation degree of PGC-1α in dopaminergic neurons-** Immunoprecipitation experiments. PGC-1α was immunoprecipitated from control and FGF21-treated cells followed by immunoblotting as described in Methods. The degree of acetylation of PGC-1α was analyzed using the anti-acetylated lysine antibody. Total amount of PGC-1α in the immunoprecipitate was analyzed using anti-PGC-1α antibodies. Values are means± SD, n=4, *p<0.05 for FGF21 vs Controls.

## ***FGF-21 increases Nampt and NAD+ levels and SIRT-1 in the dopaminergic neurons***

SIRT-1 is a deacetylase, it shifts acetyl groups from PCG-1α and consequently can active it. This reason led us to investigate if FGF-21 could influence also the SIRT-1 activity. Results of immunoblot experiments with an anti-SIRT-1 antibody, shown that

stimulation of cells for 24 hours with 50ng/ml FGF-21 increased SIRT-1 levels (Fig. 8). FGF-21 treatment, after 24 hours is also able to increase NAD+ levels in human dopaminergic neurons (Fig. 9). $NAD^+$ that are important in redox reactions and ATP production in the cell and is the important co-factor to active SIRT-1. We measure NAD+ levels using the NAD+/NADH assay.

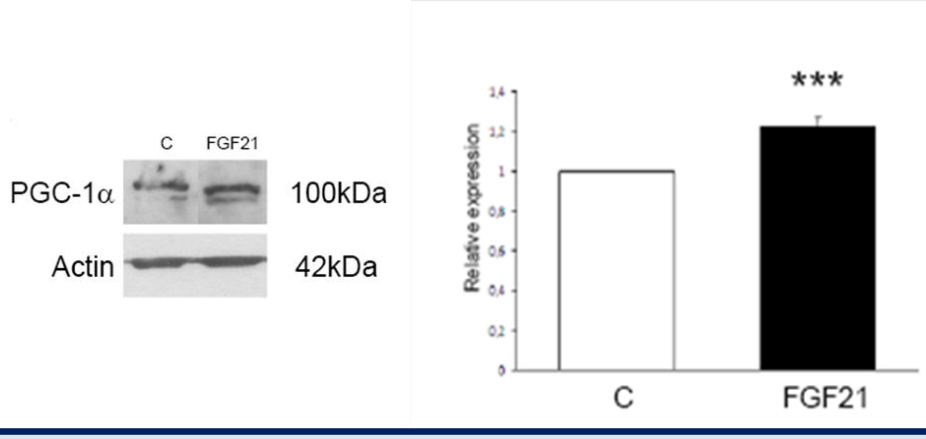


**Fig 8- FGF-21 increases SIRT1 levels in dopaminergic neurons-** Immunoblots. β-actin was used as a control. The levels of SIRT1 were increased by FGF21. Left, immunoblots. Right, quantifications using ImageJ. Values are means± SD, n=4, ***p<0.001 and *p< 0.05 for FGF21-treated vs Controls.

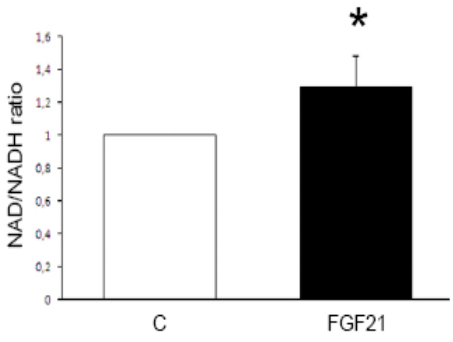


**Fig. 9- FGF-21 increases NAD/NADH levels in dopaminergic neurons-** The ratio of NAD+ to NADH in the dopaminergic neurons was measured as described in Methods. FGF21 increased NAD+ levels in the neurons. Values are means± SD, n=4, *p< 0.05for FGF21 vs Controls.

We also investigate the Nampt levels and we found it increased. Nampt is the enzyme that catalyzes the first step of $NAD^+$ biosynthesis, in fact it is the rate-limiting enzyme of $NAD^+$ production. These data shown that FGF-21 increasing Nampt and $NAD^+$ levels is able to active SIRT-1 a consequently PGC-1α in human dopaminergic neurons (fig. 8-9-10).

To confirm these data we also incubate the cells, previously treated with FGF-21, with the SIRT-1 inhibitor nicotine amide (NAM). NAM induces the disactivation of PGC-1α because, blocking the activity of SIRT-1, increases the degree of lysine acetylated of PGC-1α. In our experiments NAM is able to counteract the FGF-21 action on PGC-1α in the human dopaminergic neurons (Fig. 12). Together these results indicate that FGF-21 acts on Nampt/NAD+/SIRT-1 pathway in the human dopaminergic neurons, with effect on the PGC-1α activation.


**Fig 10- FGF-21 increases Nampt levels in dopaminergic neurons-** Immunoblots. β-actin was used as a control. The levels of Nampt were increased by FGF21. Left, immunoblots.Right, quantifications using ImageJ. Values are means± SD, n=4, ***p<0.001 and *p< 0.05 for FGF21-treated vs Controls.

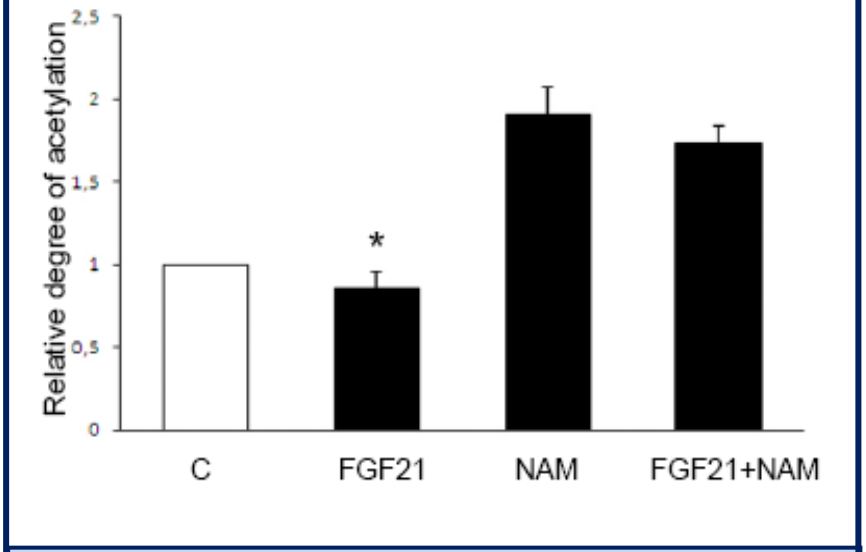


**Fig. 11: The SIRT1 inhibitor NAM increases the acetylation degree of PGC-1α**-Immunoprecipitation experiments. Cells were stimulated with FGF21 for 24h in the absence or presence of 20 μM NAM that is an inhibitor of SIRT1. The degree of acetylation of PGC-1α was analyzed as above. Note an increase in acetylated PGC-1α due to SIRT1 inhibition by NAM. Values are means± SD, n=e, *p<0.05 for FGF21vs Controls.

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## ***FGF21 increases mitochondrial antioxidants in human dopaminergic neurons***

We have previously showed that the levels of the mitochondrial antioxidants, SOD2 and Trx2 were increased in the SN of PGC-1a transgenic mice compared with wild-type animals. Likewise, the mitochondrial enzyme COXIV was also elevated in the transgenic mice compared to controls (Mudò et al 2012). The increase in protein levels in the PGC-1α transgenic mice was accompanied by an enhanced gene expression, as shown for SOD2 and COX IV, this suggest that overexpression of PGC-1α leads to changes in gene expression for a particular set of proteins in the SN with a protective function in cell

stress. So here we investigate whether these antioxidants were also increased in human dopaminergic neurons after FGF-21treatments. Data prove that treatment with FGF-21 strongly upregulates both SOD2 and Trx2 (Fig. 12). This suggest that the fibroblast growth factor-21 can contribute to neuroprotection against oxidative stress in the dopaminergic neurons, and its action is mediate by the activation of PGC-1α, that induces an increase of two important antioxidant mitochondrial enzymes antioxidants.

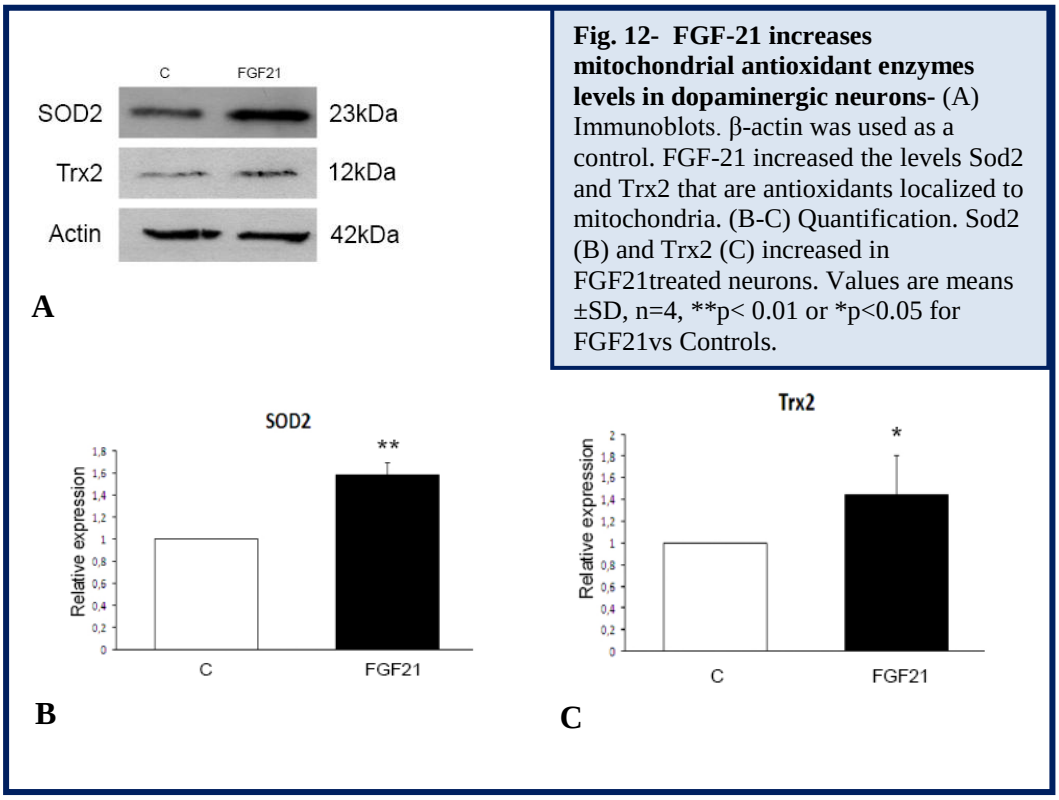



**Fig. 12- FGF-21 increases mitochondrial antioxidant enzymes levels in dopaminergic neurons-** (A) Immunoblots. β-actin was used as a control. FGF-21 increased the levels Sod2 and Trx2 that are antioxidants localized to mitochondria. (B-C) Quantification. Sod2 (B) and Trx2 (C) increased in FGF21treated neurons. Values are means ±SD, n=4, **p< 0.01 or *p<0.05 for FGF21vs Controls.

## ***FGF-21 stimulates mitochondrial respiratory capacity of human dopaminergic neurons***

To understand if FGF-21 could have effects on mitochondrial functions of human dopaminergic neurons we performed a real-time analysis using Searose equipment as described above. Our results shown that FGF-21 increased basal respiration and higher maximal respiratory capacity in treated cells as compared to controls (Fig 13). These data jointly with results about antioxidants, indicate that FGF-21 in human dopaminergic neurons can intensify the mitochondrial activity against oxidative stress.

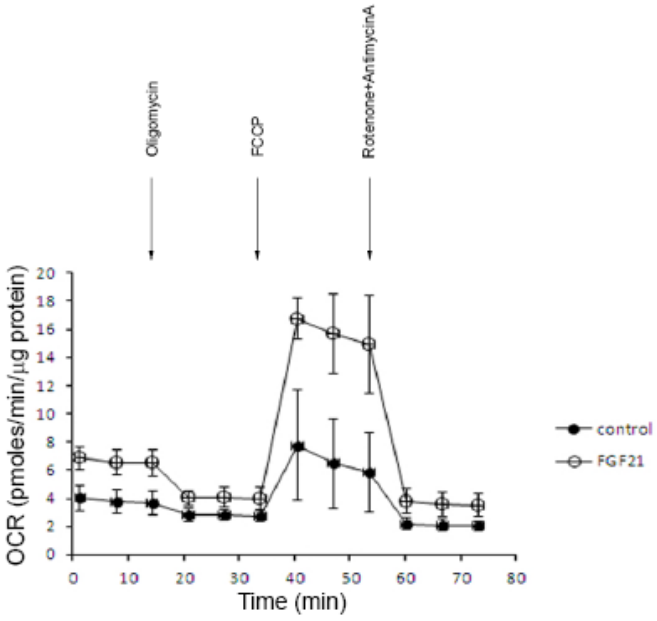



**Fig.13- FGF-21 enhances the mitochondrial respiratory activity-** The mitochondrial respiratory capacity in human dopaminergic neurons was measured in real-time using the Seahorse equipment as described in Methods. The basal as well as the maximum respiration was improved in cells treated with FGF-21 for 24h.

In the light of these results, we wanted to investigate whether treatment with FGF-21 could affect the number of mitochondria and their biogenesis. To this end we evaluated mitochondrial markers such as the mitochondrial transcription factor A (TFAM) and the cytochrome oxidase IV (COX IV). Both these markers levels were unchanged in FGF-21 treated neurons as compared with controls (Fig. 14).

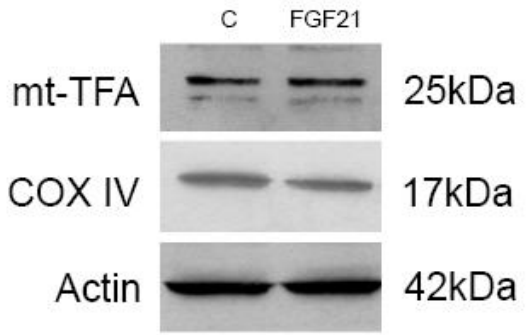
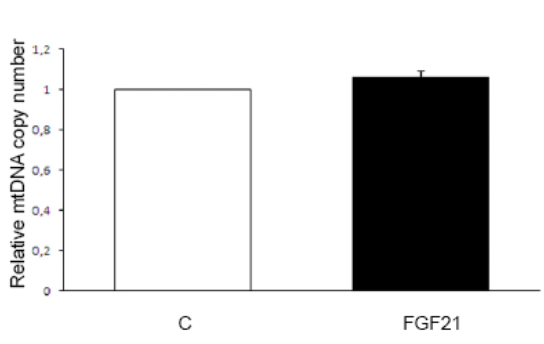
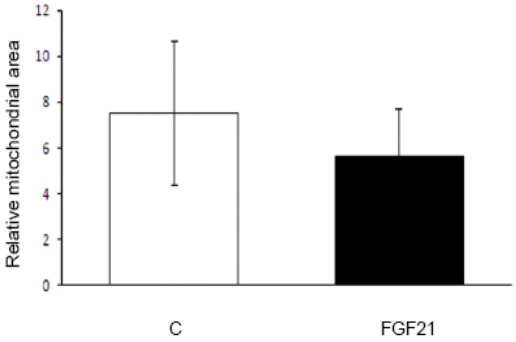



**Fig.14- FGF-21 does not alter mitochondrial biogenesis in dopaminergic neurons-** Immunoblots. β-actin was used as control. The mitochondrial proteins mt-TFA and COX IV were no significantly changed in dopaminergic neurons treated with FGF-21.

We also estimated the ratio of mitochondrial DNA through quantitative PCR. The content of mitochondrial DNA was not changed compared with nuclear DNA, this indicate that the number of mitochondria in cells war invariated, after treatment with FGF-21. Using Electron Microscopy (EM) we studied also the mitochondrial morphology, we substantiate previous data, in fact the measurement of the relative mitochondrial area from EM sections not showed significant changes between treated with FGF-21, and not treated neuronal samples (Fig 15). On the basis of these observations we can deduce that the increased mitochondrial activity on FGF-21 treated human dopaminergic neurons, is not related with an increased number of these organelles in the cells.




A




B

**Fig.15- A-B: FGF-21 does not increase the number of mitochondria in dopaminergic neurons-** The mitochondrial DNA (mt-DNA) copy number was analyzed using quantitative PCR as described in Methods. There was no change in the relative ratio of mt-DNA to nuclear DNA reflecting an equal number of mitochondria in control and FGF21-treated dopaminergic cells. Mitochondrial surface area. Control and FGF21 treated cells were analyzed by electron microscopy and the relative mitochondrial area was calculated from EM pictures as described in Methods. There were no significant changes in the mitochondrial surface area between control and FGF-21 treated cells.

Mitochondrial surface area. Control and FGF-21 treated cells were analyzed by electron microscopy and the relative mitochondrial area was calculated from EM pictures as described in Methods. There were no significant changes in the mitochondrial surface area between control and FGF-21 treated cells.

## ***FGF-21 is expressed in the midbrain and microglia cells***

Another important aim for our study was to investigate about the presence of FGF-21 in the brain. To this end we performed immunoblotting experiment using samples of adult rodent brain and antibodies against FGF-21. Data showed that FGF-21 is expressed in several brain areas such as Hippocampus, cortex, striatum and substantia nigra, were reside the cell body of dopaminergic neurons (Fig. 16).

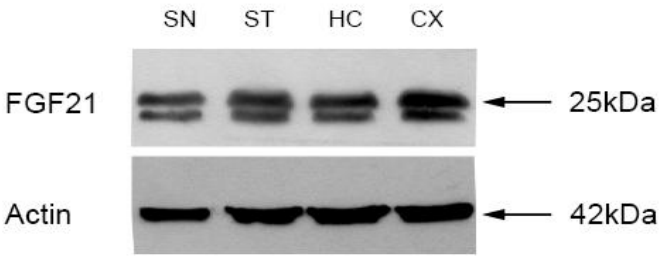



**Fig. 16- FGF-21 is expressed in several Brain areas-** Brain regions from adult mice were dissected and subjected to immunblotting using anti- FGF-21 antibodies. β-actin was used as control. FGF-21 is present in all brain areas. Two bands are detected representing the precursor and processed (mature) form of FGF21. SN, substantia nigra; ST, striatum; HC. Hippocampus; Cx, Cortex.

Immunohistochemical analyses performed with 14 μm sections of midbrain region, particularly on the substantia nigra, showed that FGF-21 is also express in these region cells (Fig 15). To understand which cell type is involved in the production of FGF-21 in the brain, we immunostained midbrain sections using markers for neurons, Tyrosine Hydroxylase (TH) to identify dopaminergic neurons, and glial cells, Glial Fibrillary Acidic Protein glial (GFAP). Data showed that neither TH-positive neurons nor GFAP-positive astrocytes expressed FGF-21 to any significant extent (data not shown). The precise localization of FGF-21 became clear after activation of the microglial cells using the excitotoxin kainic acid as shown in figure 17. Kainic acid is structurally analog to the neurotransmitter glutamate that produces neuroexcitatory agonist effects at the kainite ionotropic receptors. The kainate receptors are non-NMDA glutamate-recognizing ionotropic receptors, and agonism by Kainic acid or glutamate produces excitation and contribution to excitatory postsynaptic currents.

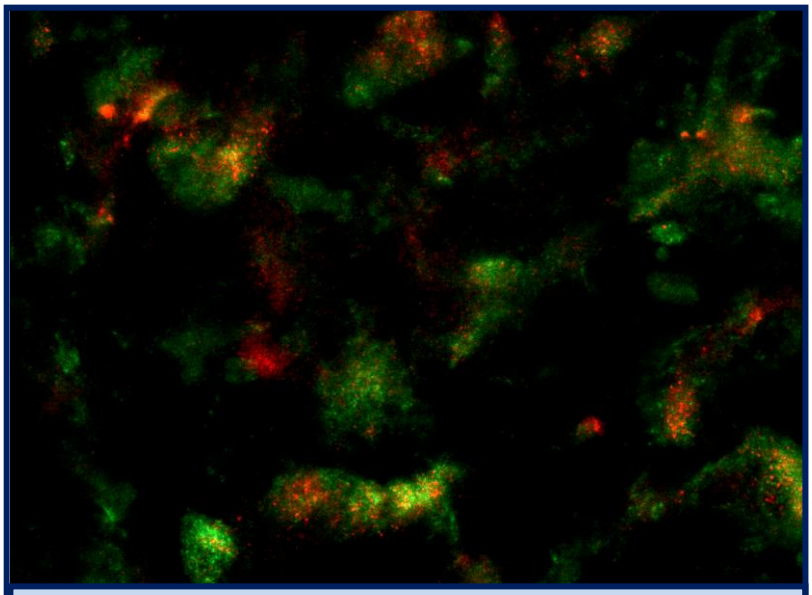


**Fig.17- FGF-21 expression in microglial cells of rat brain-** Immunohistochemical analisys of FGF-21 (green) show its expression in microglial cells CD-11 positive (red spots) following treatment with Kainic acid.

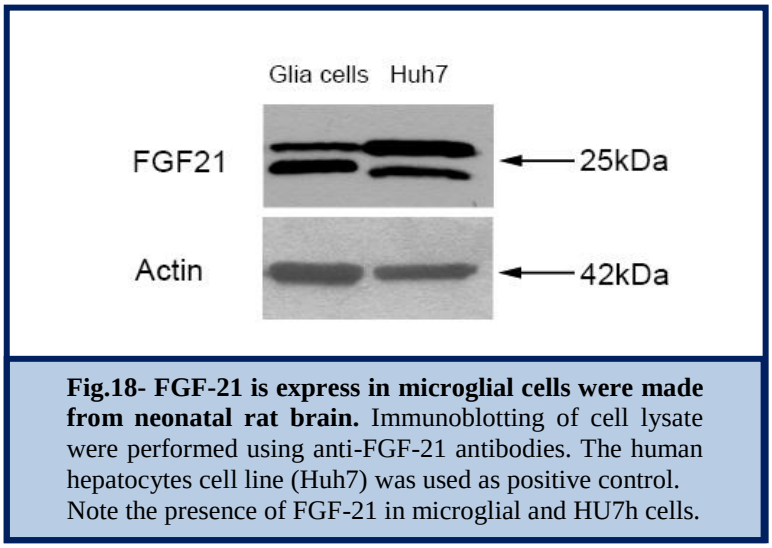



**Fig.18- FGF-21 is express in microglial cells were made from neonatal rat brain.** Immunoblotting of cell lysate were performed using anti-FGF-21 antibodies. The human hepatocytes cell line (Huh7) was used as positive control. Note the presence of FGF-21 in microglial and HU7h cells.

Agonism of the kainate receptors by sufficient concentrations of Kainic acid produces excitotoxicity, and Kainic acid is widely employed as a tool for inducing excitotoxic seizures. So the presence of this neurotoxin in brain induced the microglial cells activation, and our data shown that FGF-21 co-localized with glial cells positively stained for Cd11b (Mac-1) that is a marker for active microglia cells in the brain (Fig. 17), and its expression increases after a prolonged treatment with the neurotoxin. To substantiate this further, microglial cells were cultured from rat brains and analyzed by immunoblotting. Results showed that microglia-enriched cultures express FGF-21 in vitro in amounts equal to those found in the human hepatocyte Huh7 cell line (Fig. 18). This data shows that microglia cells can produce FGF-21 in the midbrain that could then act on the dopaminergic neurons to enhance neuronal viability.

# ***Discussion***

Fibroblast Growth Factor-21 is a member of the FGFs family, particularly FGF-19 endocrine sub-family. It is expressed (in rodents, non-human primates and in humans) predominantly in the liver, but also in the adipose tissue, pancreas and muscle. It has been studied mainly for its effects on metabolism and cell responses in peripheral cells; in fact FGF-21 plays a role in balance energy, exercising hormone-like effects by regulating fatty acids metabolism and by regulating in the uptake of glucose, (participating in the regulation of expression of insulin and glucagone). Furthermore it also drew the attention of researchers because it can be involved in a several kind of disease, in different tissues (liver, hearth, pancreas, kidney). It may be altered, in some cases, and could be considered as a diagnostic or prognostic marker of diseases (Woo Y. et al 2013). Moreover FGF-21 exerts its functions, also under stress conditions, in several tissues inducing the expression of PGC-1α (liver, adipose tissue). Although it has been widely studied literature is poor on expression and functions of FGF-21 in the brain.

PGC-1α is a transcriptional co-activator, and it is considered a major regulator of mitochondrial biogenesis, furthermore plays an important role regulating gene expression of antioxidant mitochondrial enzymes, it is express in several tissues including CNS. Importantly our research group, lately demonstrate, that PGC-1α is able to confer neuroprotective effects in dopaminergic neurons, on Parkinson's Disease animal model (Mudò et. al 2012). In the present work we investigated about possible interactions between FGF-21 and PGC-1α, in human dopaminergic neurons. To this end we used human neuronal precursors, grown in appropriate medium and conditions to induce differentiation into dopaminergic neurons, we treated cells with an adequate dose of FGF-21, and after 24 hours we found an increase of PGC-1α protein expression; we also found enhanced the PGC-1α gene promoter activity, this suggest that the FGF-21 action on PGC-1α is not restricted to metabolic tissues but can act, regulating both the transcription and the production of the protein, also in the brain.

We also observed that the increase of PGC-1α is probably related with the increase of mitochondrial activity in neurons. In fact after the FGF-21 treatment we found increased levels of antioxidant mitochondrial enzymes such as Trx2 and Sod2, indicating an enhanced mitochondrial activity, these data also confirm a previous study in which,

using transgenic mice overespressing PGC-1α, we demonstrate the ability of PGC-1α to induce the expression of molecules against oxidative stress, and this action can be considered part of its neuroprotective effect. PGC-1α also takes part in protection against cell stress and oxidative damage accompanying human metabolic disorders and degenerative diseases (St-Pierre et al., 2006). As shown in vivo, the increase in mitochondrial antioxidants by PGC-1α was accompanied by cell protection of dopaminergic neurons against the neurotoxin MPTP induced oxidative stress (Mudò et al., 2012). Together these results show that PGC-1α is an important factor in regulation of dopaminergic neuron viability and that growth factors like FGF-21 may act via the induction of PGC-1α and its downstream pathways in these neurons, participating to these beneficial effects. The activity of PGC-1α is regulated through transcriptional and post-transcriptional events. The most effective and frequent adjustment for this protein is operated by the de-acetylase SIRT-1. De-acetylation of specific lysine residues of PGC-1α, makes it more accessible the binding site to transcriptional factors, then activates the protein and capable of carrying out its functions. In our previous study we found that, the compound resveratrol acting via the SIRT1/PGC-1α can also stimulate Sod2 and Trx2 levels both in cultured neurons and in the brain (Mudò et al., 2012).

Here we were able to demonstrate by which mechanism FGF-21 can regulate the PGC-1α activity. In human dopaminergic neurons, the treatment with FGF-21 also induces an increase of SIRT-1 levels, so FGF-21 can induce antioxidant activity trough activation of SIRT1/PGC-1α pathway. In fact we further observed that the levels of $NAD^+$ ,the cofactor for SIRT-1, increased in the dopaminergic neurons following FGF-21 treatments. FGF-21 also elevated the nicotinamidephosphoribosyltransferase (Nampt) enzyme levels, that is rate limiting in the biosynthesis of $NAD^+$ from nicotinamide (NAM) (Revolle et al., 2004; Yang et al., 2007). Previous studies carried out in vascular smooth cells, pancreatic β-cells and in fibroblasts, showed that the enzyme Nampt (also called pre-B cell colony-improving factor (PBEF) can be induced by visfatin or by reduction of nutrients or by cytokines and that Nampt is involved in regulating metabolic responses, inflammation, and cell differentiation in peripheral cells (Garten et al., 2009). With ours data we demonstrate that the Nampt-SIRT1 pathway is also active in neurons and it can be regulated by FGF-21 signaling, this could reveal of FGF-21 a role response to inflammation also in the Brain. These results in dopaminergic neurons add to previous data showing the involvement of Nampt in the biological events described before. Given

its role in the activation of SIRT-1 in dopaminergic neurons it would be interesting to study the regulation of Nampt in animal models of PD and after treatment with neurotrophic factors.

Gerhart-Hines et al in 2007 demonstrated that FGF-21 is involved in regulation of mitochondrial functions by the SIRT1/ PGC-1α in muscle cells. To establish if FGF-21 treatment could be able to increase in dopaminergic neurons the mitochondrial activity, we performed a real-time assay to measure the mitochondrial respiratory capacity, using the Seahorse equipment. We observed an increased mitochondrial activity, in line with previous data mentioned above. Though not formally proven, we suspect that this effect is due to the activation of PGC-1α by FGF-21 in these neurons. Subsequently we investigate if FGF-21, as a growth factor, could alter the number of mitochondria in the cell. For this purpose we analyzed the levels of two mitochondrial markers: COX IV and mt-TFA respectively, protein of the respiratory chain, and transcriptional factor regulator of mitochondrial biogenesis; and the mitochondrial DNA copy number using PCR. Our results do not show significant changes in protein levels compared to controls, or in the number of the copy of mt-DNA, as compared to the ratio of nuclear DNA. Together these data indicate that the increased mitochondrial activity is related to the induction of FGF-21, rather than to increase the number of organelles.

It is known that some members of FGFs family are expressed in the brain, like FGF-1 FGF-2 but also FGF-13 and FGF-14 and have a role in development and in some cases in neuronal diseases (Mudò et al., 2009; Itoh and Ornitz, 2011; Lahti et al., 2012). So far little is known about the expression of FGF-21 in brain or in brain cell metabolism. Because of this we wanted to deepen the knowledge of FGF-21 expression in the brain. We performed immunoblotting experiments that shows that FGF-21 in expressed to an appreciable amount in various brain regions of adult mouse, such as cortex, Striatum, Hippocampus and Substantia nigra. We used an immunohistochemical approach to understand which cell type could express the growth factor. Data highlight that FGF-21 was mainly localized to microglial cells, overall in the SN where, moreover, the FGF-21 expression is increased after neurotoxic treatment with Kainic acid. This may suggest a role of FGF-21 in in response to excitotoxic damage. These observations open up possibilities for new considerations and future studies aimed to understanding what are possible the mechanisms underlying the involvement of FGF-21 in response to

excitotoxic events, overall in the substantia nigra which is the brain area most damaged in Parkinson's disease.

Alterations of growth factors are strongly related to metabolic diseases, but also may also prevail in neurodegeneration (Patrone et al, 2013). The expression of FGF-21 is greatly increased during prolonged fasting, it has several effects on the metabolism, and is involved in many metabolic diseases; it is able to pass the blood brain barrier, so can be also involved in neurological disorders like PD, or others neurodegenerative diseases. In will be interesting in the future also to investigate about its rate of involvement in these kind of disorders.

# ***Conclusions***

We have shown here, that human dopaminergic neurons respond to treatment with Fibroblast Growth Factor 21, increasing the expression and the activity of the transcriptional co-activator PGC-1α via SIRT-1, and enhancing the mitochondrial respiratory activity, giving greater antioxidant activity. So these effects do not depend by the numerical increase in these organelles, rather than by the direct action of FGF-21 on PGC-1α. Moreover we observed that FGF-21 is express in both basal and stress conditions in several brain areas, particularly in microglial cells, including substantia nigra. These results are interesting and suggest that microglia-derived FGF-21 may influence dopaminergic and possible also other neurons in the brain. It will be important to study in more detail the role of FGF-21 in microglial cells and in neuroimmunological responses in the brain and in models of PD. These data together give us more knowledge about the physiology of dopaminergic neurons, and emphasizes and supports the foundation for understanding of the neuroprotective mechanisms based on PGC-1α activity, and open new scenery for future studies, with a possible therapeutic approach of neurodegenerative diseases such as PD.

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