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SHORT-TERM DIABETES IN THE BRAIN: EFFECTS ON NEUROACTIVE STEROIDS, CHOLESTEROL HOMEOSTASIS, AND MITOCHONDRIAL FUNCTIONALITY

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Abbreviations

Abbreviations

36B4: ribosomal protein 36B4
3α-diol: 5α-androstane-3α,17β-diol
3β-diol: 5α-androstane-3β,17β-diol
3β-HSD: 3β-hydroxysteroid dehydrogenase
3α-HSOR: 3α-hydroxysteroid oxidoreductase
5α-R 1: 5α-reductase type 1
7α-OH: 7α-hydroxycholesterol
7β-OH: 7β-hydroxycholesterol
7-keto: 7-ketocholesterol
17β-HSD: 17β-hydroxysteroid dehydrogenase
24(S)-OH: 24(S)-hydroxycholesterol
25-OH: 25-hydroxycholesterol
27-OH: 27-hydroxycholesterol
ABCA1: ATP-binding cassette A1
ABCG1: ATP-binding cassette G1
ACAT: cholesterol acyl transferase
AD: Alzheimer's disease
ANTs: nucleotide translocators
ApoE: Apolipoprotein E
AR: androgen receptors
CEs: cholesteryl esters
CHO: Chinese hamster ovary cell line
CNS: central nervous system
CSF: cerebrospinal fluid
CYP27A1: cholesterol 27-hydroxylase
CYP46A1: cholesterol 24-hydroxyase
CYP19: aromatase
DE: diabetic encephalopathy
DHCR24: 24-deydrocholesterol reductase;
DHCR7: 7-dehydrocholesterol reductase
DHDOC: dihydrodeoxycorticosterone
DHEA: dehydroepiandrosterone
DHP: dihydroprogesterone
DHT: dihydrotestosterone
EAE: experimental autoimmune encephalomyelitis
ERs: estrogen receptors
ER: endoplasmic reticulum
ETS: electron transport system
GABA-A/B: gamma aminobutyric acid-A/B
GLUTs: glucose transporters
GSH: glutathione
HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A
HMG-CoA R: 3-hydroxy-3-methylglutaryl coenzyme A reductase
HSL: hormone-sensitive lipase
IL: interleukin
IMM: inner mitochondrial membrane
i.p.: intra-peritoneal
ISOPREG: isopregnanolone

LC-MS/MS: liquid chromatography-tandem mass spectrometry analysis
LDLR: Low density lipoprotein receptor
LXRs: Liver X receptors
MAM: mitochondria-associated endoplasmic reticulum membrane
mt-COX II: mitochondrial-cytochrome C oxidoreductase
MS: multiple sclerosis
NDD: neurodegenerative disorders
NCEH: neutral cholesteryl esters
NMDA: N-Methyl-D-aspartate
NO: nitric oxide
NRF: nuclear respiratory factors
OMM: outer mitochondrial membrane
OXPHOS: functional subunits of respiratory chain complexes
P450scc or CYP11A1: cytochrome P450 side chain cleavage
PD: Parkinson's disease
PGC-1α: proliferator-activated receptor gamma-1 alpha
PKA: cAMP-dependent protein kinase A
PNS: peripheral nervous system
PR: progesterone receptors
PREG: pregnenolone
PROG: progesterone
ROS: oxygen reactive species
RNS: reactive nitrogen species
RR-MS: relapsing remitting multiple sclerosis
SOAT1: sterol O-acyltransferase 1
SOD: superoxide dismutase
SREs: Sterol-responsive elements
SREBPs: Sterol regulatory binding elements
SCAP: SREBP cleavage-activating protein
StAR: steroidogenic acute regulatory protein
STZ: streptozotocin
T: testosterone
TBARS: thiobarbituric acid reactive substances
TCA: tricarboxylic acid cycle
TFAM: mitochondrial transcription factor A
THP: tetrahydroprogesterone
TNF-α: tumor necrosis α
TSPO: 18 kDa translocator protein
UCP2: Uncoupling protein 2
VDAC2: voltage-dependent anion channel 2

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Summary

Diabetes may induce neurophysiological and structural changes in the central nervous system (i.e., diabetic encephalopathy). Neuroactive steroids (i.e. molecules derived from cholesterol which exert their actions in the nervous system directly or after metabolization) are key regulators of the central nervous system and are affected in several neuropathological disorders. As previously observed by our laboratory, three months of diabetes modifies the levels of neuroactive steroids in different brain regions of an experimental model of diabetic rat (i.e. raised diabetic by streptozotocin). On this basis, we explored whether neuroactive steroid levels may be already affected after one month (i.e., a period in which diabetic encephalopathy is already apparent), in two brain regions such as hippocampus and cerebral cortex. By liquid chromatography-tandem mass spectrometry analysis we observed that the levels of several neuroactive steroids, such as pregnenolone, progesterone and its metabolites (i.e., tetrahydroprogesterone and isopregnanolone), testosterone and its metabolites (i.e., dihydrotestosterone and 3α-diol) in the hippocampus and cerebral cortex were altered. Interestingly these brain changes were not fully reflected by the plasma level changes, suggesting that early phase of diabetes directly affects steroidogenesis and/or steroid metabolism in these brain areas. To further explore this finding we analyzed crucial steps of the steroidogenic machinery, like for instance the gene expression of steroidogenic acute regulatory protein (i.e., molecule involved in the translocation of cholesterol into mitochondria) and cytochrome P450 side chain cleavage (i.e., enzyme converting cholesterol into pregnenolone). In addition, we analyzed cholesterol homeostasis (i.e., synthesis, trafficking and metabolism) as well as mitochondrial functionality, a key organelle in which the limiting step of neuroactive steroid synthesis takes place. Data obtained indicate that short-term diabetes alters steroidogenic machinery (e.g. Star and P450scc) in the hippocampus. On the contrary, in the cerebral cortex only the gene expression of StAR was down-regulated by short-term diabetes after one month, while P450scc remained unchanged. In addition, an impairment of cholesterol homeostasis as well as mitochondrial dysfunction, even if with different characteristics, was present in hippocampus and cerebral cortex. In conclusion, data here reported indicate that short-term diabetes modifies the levels of neuroactive steroids in hippocampus and cerebral cortex. These changes, depending on the

brain structures considered, are associated with alteration of steroidogenic machinery, cholesterol homeostasis and mitochondrial dysfunction.

Introduction

Steroids, not only peripheral messengers

In the classical view, steroid hormones are lipid molecules synthesized exclusively in peripheral organs such as adrenal cortex or gonads which exert their actions in the periphery. Steroids hormones exert their regulatory properties through interaction with nuclear receptors (Stumpf et al., 1975, Brinton et al., 2008, Melcangi et al., 2008). However, steroids are also able to activate different membrane or cytoplasmic signaling pathways. Initial observations suggested that nuclear receptors distribution in the CNS was mainly restricted to the hypothalamic region. These receptors (now named classical steroid receptors) are localized in the cytoplasm and, when activated by binding to the hormone, translocate into the nucleus where they exert a regulatory action on the genome (Yamamoto, 1985). For example, progesterone (Blaustein, 2003), estrogen (Shupnik, 2002), androgen (Cato and Peterziel, 1998), glucocorticoid and mineralocorticoid receptors are included in the category of classical steroid receptors (McEwan et al., 1997). The activation of these receptors may explain the medium- and long-term effects of steroid hormones (such as the regulation of the secretion of hypophyseal hormones, or the sexual differentiation of brain circuits). Moreover, additional studies indicate that steroids might also induce short-term effects (i.e. effects that take place in seconds or minutes), thus suggesting the existence of other receptors (i.e. the so-called non-classical steroid receptors) located within the membrane and thus able to act as mediators of short-term actions. These receptors include GABA type A and type B receptors (GABA-A receptor, GABA-B receptor), serotonin type 3 (5-HT3), N-Methyl-D-aspartate (NMDA), alpha-amino-3hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainite receptor, and an atypical intracellular receptor like the sigma 1 (Melcangi et al., 2005).

Neurosteroids and neuroactive steroids

The lipophilic nature of steroids makes them able to diffuse across the cell membranes and to bind to steroid receptors in tissues. In the last 70 years, several studies have demonstrated that these molecules exert a wide array of functions on the central (CNS) and peripheral nervous system (PNS), controlling processes such as sexual differentiation of the brain, reproduction, behavior, and memory (McEwen, 1981, Fink et al., 1991, McEwen, 1994).

The mechanisms by which steroids exert their effects on the nervous system were considered to be the classical endocrine mechanisms. In this concept, steroids are produced by endocrine glands such as the adrenals and gonads, secreted into the bloodstream, and then, after crossing the blood-brain barrier, they regulate CNS functions. However, the nervous system is not only a target of endocrine effects exerted by hormonal steroids released by peripheral steroidogenic tissues. Indeed, in the 1981 Baulieu and co-workers showed that CNS can itself synthesizes the steroids (Corpechot et al., 1981). Then, Corpechot et al. (1983) demonstrated the presence of significant amount of PREG, dehydroepiandrosterone (DHEA) and their sulfate esters (PREGS and DHEAS) in the mammalian brain after 15 days of adrenalectomy and orchiectomy. Baulieu et al. (1998) called these molecules "neurosteroids", which are steroids directly synthesized by neurons and glial cells that have the ability to regulate the activity of the nervous system (Baulieu, 1999), with autocrine or paracrine actions (Melcangi et al., 2008). However, the consolidation of the concept of neurosteroids has required several investigations performed in various laboratories using different animal species (Baulieu, 1999, Mensah-Nyagan et al., 1999, Compagnone and Mellon, 2000, Schumacher et al., 2003). These investigations, which significantly increased the basic knowledge on neurosteroids, have also allowed a strict definition of the term neurosteroids with specific identification criteria. Moreover, to be qualified as neurosteroids, the candidate steroidal molecule must persist in a substantial amount in the nervous system after removal of the peripheral or traditional steroidogenic glands such as the adrenals and gonads (Baulieu 1999). Furthermore, all these observations indicate that the nervous system is a target for two different pools of steroids, one coming from the peripheral glands (i.e. steroid hormones) and the second one originating directly in the nervous system (i.e. neurosteroids). In many circumstances, it is difficult to discriminate whether the steroid effect is due to their *in situ* synthesis, to peripheral hormones, or to an enzymatic conversion of steroids in metabolites which are more active and in some cases which act through different mechanisms of action. For this reason, some investigators in this field use now the term "neuroactive steroids" (Paul and Purdy, 1992). More recently, this concept was expanded including the synthetic steroids that are able to regulate several neuronal functions (Melcangi et al., 2008).

Neuroactive steroids play an important role as regulators of neuronal survival under neurodegenerative conditions by binding to the classical steroid receptors. For instance,

testosterone (T) and progesterone (PROG) have been shown to be neuroprotective in different experimental models of neuronal injury including hippocampal excitotoxicity, substantia nigra degeneration, and experimental forebrain ischemia. Moreover, these steroids are protective in affective disorders (Sousa et al., 2000, Garcia-Segura et al., 2001). Due to their ability to control homeostatic parameters, and to protect the nervous system during crucial pathophysiological mechanisms (i.e. neurodegenerative processes and signaling pathways involved in neuronal cell death), over the two past decades, neuroactive steroids have received a great amount of attention (Charalampopoulos et al., 2006, Dubrovsky, 2006, Melcangi and Garcia-Segura, 2006, Melcangi and Panzica, 2006)

Synthesis and metabolism of neurosteroids

The formation of neurosteroids has been reported in the nervous system by various molecular and biochemical studies, which have revealed in neurons and/or glial cells the expression of 18 kDa translocator protein (TSPO), the steroidogenic acuter regulatory protein (StAR) and of several key steroid-synthesizing enzymes such as cytochrome P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450c17 (P450c17), 5α-reductase (5α-R), 3α-hydroxysteroid oxidoreductase (3α-HSOR), 17β-hydroxysteroid dehydrogenase (17β-HSD) and aromatase (CYP19) (Baulieu, 1999, Mensah-Nyagan et al., 1999, Compagnone and Mellon, 2000, Schumacher et al., 2003).

StAR and TSPO

Steroidogenesis is a complex multi-enzyme process where cholesterol is converted to biologically active steroid hormones through a highly compartmentalized sequence of reactions, which implies as the first step the translocation of cholesterol from the cytoplasm to the mitochondrial membrane. This is a limiting step hormonally mediated by the steroidogenic acute regulatory protein (StAR) (Lavaque et al., 2006a, 2006b).

Previous findings have proposed the 37 kDa isoform of StAR as a precursor of the functional or mature, intra-mitochondrial protein of 30 kDa. More recent observations indicate that the 37 kDa StAR acts on the outer mitochondrial membrane (OMM). However, it is rapidly transported into mitochondria where it is cleaved generating a mature 30 kDa intra-mitochondrial StAR protein that, after phosphorylation, acts on the inner mitochondrial membrane (IMM) for the transfer of the majority of cholesterol (Artemenko et al., 2001,

Miller, 2013, Manna et al., 2016). This transporter predominantly mediates the rate-limiting step in steroid biosynthesis (i.e. the transport of cholesterol) from the outer to the inner mitochondrial membrane. However, the precise mechanism by which cholesterol is transported to mitochondria for steroidogenesis remains unknown; furthermore, considerable evidence suggests that a dynamic protein complex is involved in this process (Arakane et al., 1998, Artemenko et al., 2001, Liu et al., 2006, Fan et al., 2010, Rone et al., 2012, Manna et al., 2013, Orlando et al., 2013, Poderoso et al., 2013, Fan et al., 2015). Among these proteins, the StAR has essentially all of the characteristics to become an acute regulator of steroid biosynthesis in steroidogenic tissues (Clark et al., 1994, Lin et al., 1995, Stocco and Clark, 1996, Arakane et al., 1998, Stocco, 2001, Miller, 2007a, b, Miller and Auchus, 2011, Miller and Bose, 2011). In accordance with this, transcriptional and/or translational inhibition of StAR, markedly decreases, but not abolishes, steroid synthesis (Clark et al., 1997, Stocco, 2001, Manna et al., 2009). This suggest that other proteins, in addition to StAR, play important roles for the intra-mitochondrial transport of cholesterol in controlling steroidogenesis (Manna et al., 2016). Indeed, several proteins have been identified (Figure 1), however their specific role must be further clarified (Bose et al., 2008, Papadopoulos and Miller, 2012, Rone et al., 2012). Among them, the peripheral benzodiazepine receptor, now called 18 kDa translocator protein (TSPO), has been the first to be identified (Papadopoulos et al., 2006a, 2006b). TSPO was initially proposed as an "acute trigger" of steroidogenesis, but it is now clear that StAR plays that role, and that TSPO is part of the molecular machine on the OMM through which StAR acts (Papadopoulos and Miller, 2012). The presence of TSPO has been also demonstrated in the CNS by Papadopoulos et al. (2006a). Moreover, it has been demonstrated its involvement in the translocation of cholesterol from the OMM to the IMM. Observations in different neuropathological conditions such as gliomas, neurodegenerative disorders and neuroinflammation induced by neurotoxins shows an up-regulation of TSPO expression (Papadopoulos et al., 2006a). Moreover, after CNS injury, the expression of TSPO is mainly restricted to microglia and astrocytes (Kassiou et al., 2005). StAR may also interact with the voltage-dependent anion channel 2 (VDAC2) at the mitochondria-associated endoplasmic reticulum membrane (MAM) prior to its translocation to the mitochondrial matrix. VDAC may also mediate hormone-trigger mobilization of cholesterol to the mitochondrial matrix (Jefcoate, 2002). Indeed, in non-steroidogenic COS-1 cells, VDAC2 knockdown reduced PROG synthesis more than 80% (Prasad et al., 2015).
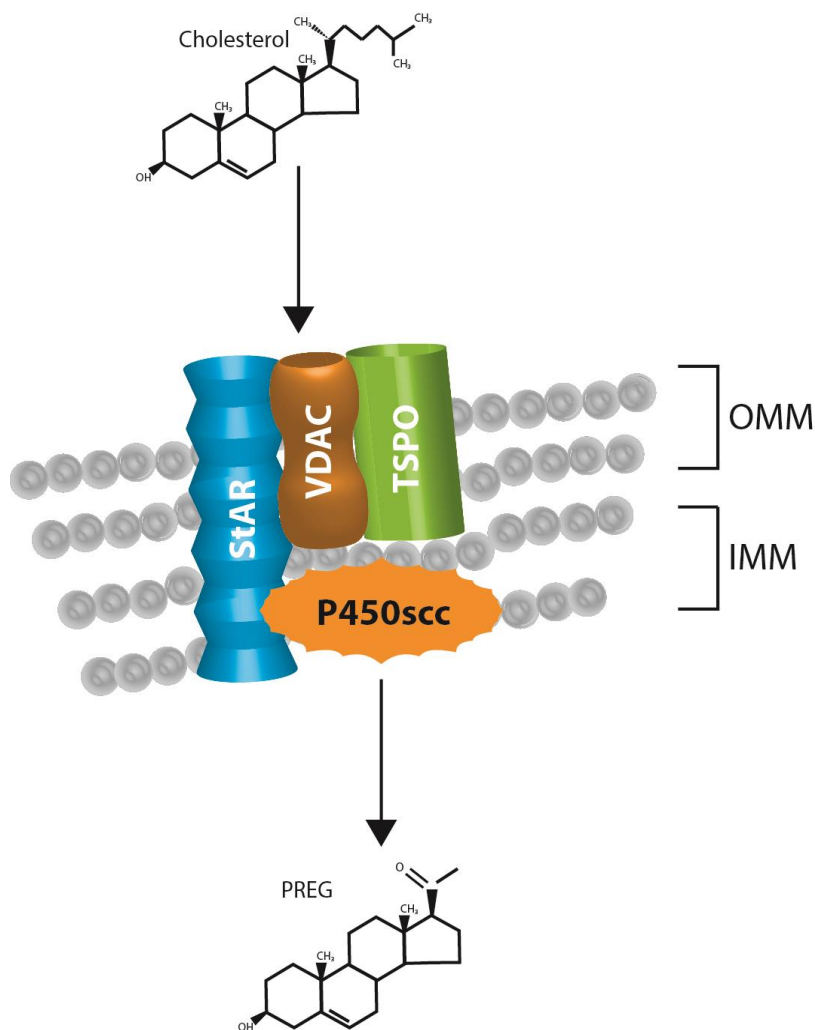
Figure 1. Transport of cholesterol into the mitochondrial compartment. Steroid acute regulation protein (StAR); voltage-dependent anion channel (VDAC); 18 kDa translocator protein (TSPO); cytochrome P450 side chain cleavage (P450scc); Pregnenolone (PREG); Outer mitochondrial membrane (OMM); Inner mitochondrial membrane (IMM). This figure represents the protein complex composed by StAR, VDAC, and TSPO that mediates the import of cholesterol through the OMM and IMM. P450scc enzyme located in the IMM is the final step of this process that converts the imported cholesterol into PREG

Recent findings have demonstrated that StAR expression is also regulated by hormone sensitive lipase (HSL), a neutral cholesteryl ester hydrolase, which converts cholesteryl esters into free cholesterol for steroid hormone production. Indeed, deficiency of HSL affects StAR and steroid levels, demonstrating that HSL plays a vital role in regulating the steroidogenic response. In accordance, the physical interaction of HSL with StAR (Figure 2) results in an elevation of the hydrolytic activity of HSL and they both facilitate trafficking of intracellular cholesterol from lipid droplets into the mitochondria (Shen et al., 2003). In addition, the inhibition of HSL activity which blocks the release of cholesterol from lipids droplets, diminishes StAR expression and steroid biosynthesis (Watari et al., 1997, Soccio et al., 2002, Zhang et al., 2002, Strauss et al., 2003).

StAR appears to be widely distributed throughout the brain, although different levels of expression have been detected between different brain areas. For instance, StAR expression seems to be restricted to very specific neuronal and astroglial populations in each brain area, although it is predominantly expressed by neurons (Sierra et al., 2003). Moreover, it has been demonstrated that the mRNA level of StAR, in the hippocampus, was approximately 1/100 of that in rat testis (Shibuya et al., 2003). The tissue-specific expression of StAR is dictated by its transcriptional regulation. Given that StAR mRNA levels rise rapidly in response to the cellular signals that also drive StAR protein expression and steroidogenesis, it is often presumed that StAR transcription and translation are tightly coupled in response to a single signaling event (Nieschlag et al., 2004, Manna et al., 2007, Manna and Stocco, 2008).
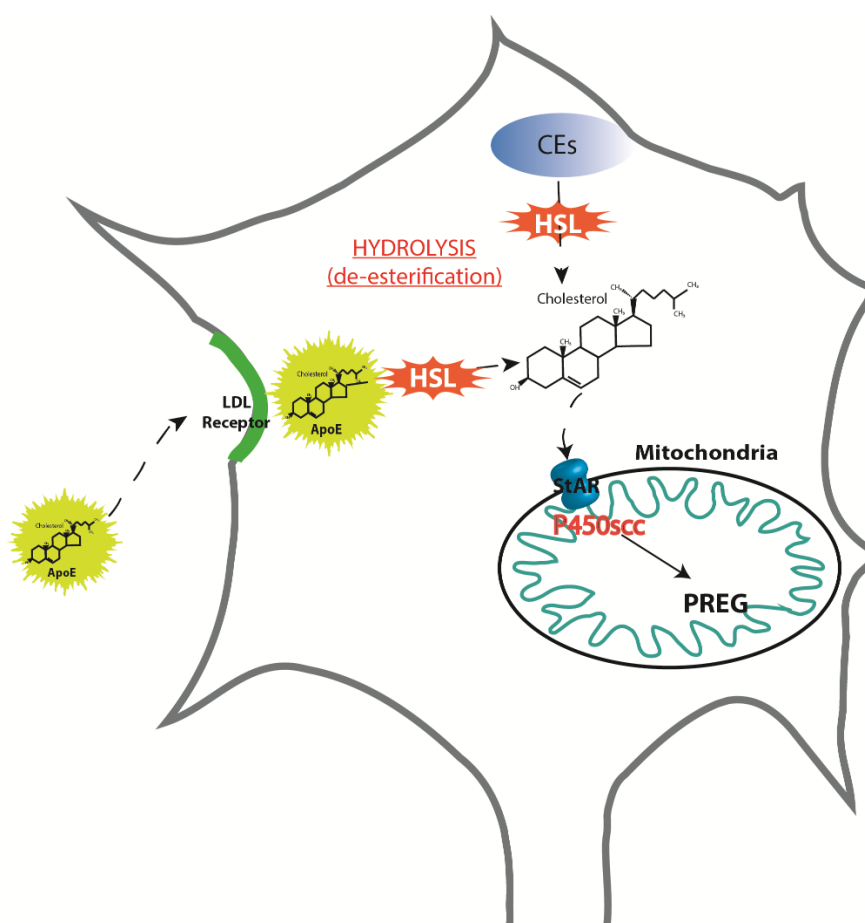
Figure 2. Role of HSL in the bioavailability of cholesterol. Cholesteryl esters or esters of cholesterol (CEs); Hormone sensitive lipase (HSL); Apolipoprotein E (ApoE); Low Density Lipoprotein (LDL) receptor; Steroidogenic acute regulatory protein (StAR); Cytochrome P450 side chain cleavage (P450scc); Pregnenolone (PREG). The cholesterol necessary for the steroidogenesis is derived from several sources. The hydrolysis of CEs stored in lipid droplets is an important source of cholesterol for optimum steroid biosynthesis. ApoE is another important source of cholesterol through the import from astrocytes. HSL is a multifunctional enzyme that is responsible of the de-esterification of CEs from lipid droplets and from ApoE.

P450scc

After the translocation into IMM, cholesterol is actively converted into PREG (Figure 3) by a mitochondrial enzyme namely cytochrome P450 side chain cleavage (P450scc) encoded by CYP11A1 gene. This is the first, rate-limiting, and hormonally regulated step in the synthesis of all steroid hormones. Thus, it is the expression of CYP11A1 gene and the respective presence of P450scc that renders a cell "steroidogenic" and able to make steroids de novo, as opposed to modifying steroids produced elsewhere, which occurs in many types of cells. This process involves three chemical reactions, the 22-hydroxylation of cholesterol, 20-hydroxylation of 22(R)-hydroxycholesterol, and oxidative scission of the C20-22 bond of 20(R),22(R)-dihydroxycholesterol (the side-chain cleavage event), to yield PREG (Hall, 1986, Tuckey and Cameron, 1993). Alternatively, soluble hydroxysterols such as 22(R)-hydroxycholesterol can enter the mitochondrion readily, without the action of StAR and its associated machinery. Catalysis by P450scc is slow, with a net turnover of approximately 6 – 20 molecules of cholesterol per molecule of P450scc per second.

As previously mentioned, transcription of CYP11A gene determines cellular steroidogenic capacity. This transcription is regulated by tissue-specific and hormonally responsive factors, and it can be induced by second messenger's system as well as protein kinase A and C acting through different CYP11A1 promoter sequences (Moore et al., 1990). Adrenal and gonadal transcription of P450scc and other steroidogenic enzymes requires the action of steroidogenic factor 1 (SF1) (Schimmer and White, 2010). By contrast, placenta expression of P450scc is constitutive, independent of SF1 (Henderson et al., 2007, Schimmer and White, 2010).

The presence of this enzyme in the white matter of the rat brain was demonstrated by immunohistochemistry (Le Goascogne et al., 1987). In addition, conversion of cholesterol into PREG was demonstrated by chemical assay in primary culture of rat glial cells (Jung-Testas et al., 1989). Finally, Shibuya et al, (2003) demonstrated that the relative levels of P450scc in the hippocampus were approximately 1/500 than in the rat testis (Shibuya et al., 2003).

P450c17

PREG is converted into DHEA by the enzymatic complex P450c17 (Figure 3), a two-step reaction that converts PREG into 17-hydroxy-PREG and DHEA. This enzyme is also responsible for the conversion of PROG into 17-hydroxy-PROG and androstenedione.

The production of DHEA in the rat brain, was suggested in early studies. Indeed, the expression of mRNA encoding P450c17 was demonstrated in cerebellum and brain stem of adult rats (Stromstedt and Waterman, 1995, Kohchi et al., 1998). Subsequently, expression and enzymatic activity of P450c17 in adult hippocampal neurons were demonstrated (Hojo et al., 2004).

17β-HSD

In all cells of CNS, 17β-HSD synthesizes androstenediol from DHEA (Jellinck et al., 2007) and testosterone (T) from androstenedione (Figure 3). Moreover, it converts also estrone into estradiol (Jellinck et al., 2006, Jellinck et al., 2007).

In humans, 17β-HSD is present in five isoforms which are expressed in several areas of body (Martel et al., 1992). Moreover, several isoforms of 17β-HSD were also detected in various rodent tissues (Normand et al., 1995). For instance, in the rat brain, the immunoreactivity for 17β-HSD is widely distributed in hippocampus, cerebral cortex, thalamus and hypothalamus (Mensah-Nyagan et al., 1999).

3β-HSD

The conversion of delta-3β-hydroxysteroids (PREG, 17OH-PREG, DHEA) into delta-3-ketosteroids (PROG, 17OH-PROG and androstenedione) is catalyzed by the enzyme 3β-HSD (Figure 3).

In human this enzyme exists in two isoforms: 3β-HSD type 1, which is essentially expressed in the placenta and 3β-HSD type 2, which is mainly expressed in the adrenal glands and gonads (Luu The et al., 1989, Rheaume et al., 1991). Moreover, through molecular cloning of the cDNA, four types of 3β-HSD were characterized in rats (Zhao et al., 1991) and six types in mice (Simard et al., 1996).

The observation of PREG conversion into PROG in homogenates of rat amygdala and septum constitutes the first result suggesting the existence of 3β-HSD in the CNS (Weidenfeld et al., 1980). The biological activity of 3β-HSD has also been detected in primary cultures of rodent oligodendrocytes and neurons (Jung-Testas et al., 1989). The expression of 3β -HSD protein and/or mRNA has been reported in various brain regions of several classes of vertebrates including fish (Mathieu et al., 2001, Sakamoto et al., 2001), amphibians (Mensah-Nyagan et

al., 1994), birds (Ukena et al., 1999) and mammals (Dupont et al., 1994, Guennoun et al., 1995, Sanne and Krueger, 1995, Furukawa et al., 1998).

5α-R

The enzyme 5α-reductase (5α-R) are a family of isozymes expressed in a wide host of organs and tissues, including the CNS. 5α-R is responsible for the transformation of testosterone and progesterone into DHT and DHP (Figure 3), respectively. In a similar manner, 5α-R converts 11-deoxycorticosterone to dihydrodeoxycorticosterone (DHDOC). Three isoforms of 5α-R, defined as type 1 (5α-R1), 2 (5α-R2) 3 (5α-R3), have been cloned in humans and rats (Andersson and Russell, 1990, Berman and Russell, 1993, Langlois et al., 2010, Paba et al., 2011). Recent findings have demonstrated that the three isozymes are present in the brain (Melcangi et al., 2011, Giatti et al., 2012a) even if it is important to highlight that 5α-R3 is still under intensive investigation. The genes encoding 5α-R1, 5α-R2 and 5α-R3 are located in chromosome 5, 2 and 4, respectively, and the two isoenzymes have different optimal pH and sensitivity to substrates (Normington and Russell, 1992, Uemura et al., 2008, Kahrizi et al., 2011). In humans, the 5α-R1 gene is predominantly expressed in the skin, notably in the pubic skin and scalp (Andersson and Russell, 1990, Jenkins et al., 1992). The 5α-R2 gene is mainly expressed in the prostate and gonads and its deletion provokes male pseudo-hermaphroditism (Andersson et al., 1991, Thigpen et al., 1993). In rats, 5α-R1 and 5α-R2 cDNAs have been cloned from a prostate library but the two genes are transcribed in distinct cells: mRNAs encoding 5α-R1 are found in the basal epithelial cells, while 5α-R2 mRNAs are localized in stroma cells (Andersson and Russell, 1990, Berman and Russell, 1993). Unlike type 1 and 2, 5α-R type 3 is thought to play a role in protein glycosylation via the dolichol phosphate pathway (Garcia-Segura and Balthazart, 2009, Giatti et al., 2012a).

The expression of 5α-R in the brain has been extensively studied (Saitoh et al., 1982, Melcangi et al., 1993, Pelletier et al., 1994, Stoffel-Wagner, 2003) and its activity has a crucial role in the control of nervous function. Indeed, the metabolites of PROG and T are involved in the regulation of neuroendocrine events, behavior, affection, learning and memory, synaptic and glial plasticity and adult hippocampal neurogenesis as well as in the response of brain tissues to injury and neurodegeneration (i.e., regulating neuronal survival, axonal regeneration and gliosis) (Kipp and Beyer, 2009, Panzica et al., 2012, Brinton, 2013, Brunton et al., 2014). It has

been suggested that 5α-R1 essentially plays a catabolic and neuroprotective role whereas 5α-R2 participates in sexual differentiation of the CNS (Traish, 2012).

To date, no genetic deficiency has been reported for 5α-R1 enzyme. In contrast, the clinical consequences of congenital 5α-R2 deficiencies are well characterized and consist in alterations of sexual differentiation (Imperato-McGinley et al., 1974, Russell and Wilson, 1994, Katz et al., 1997, Imperato-McGinley, 2002, Imperato-McGinley and Zhu, 2002, Sasaki et al., 2003a, Sasaki et al., 2003b). Mutations in 5α-R3 have also been described, and are associated with mental retardation and visual disturbances (Cantagrel et al., 2010, Morava et al., 2010, Kasapkara et al., 2012).

Aromatase

The conversion of androgens into estrogens (i.e., androstenedione and testosterone) is catalyzed by aromatase (Figure 3). The activity of this enzyme occurs in various tissues including the placenta (Fournet-Dulguerov et al., 1987), ovary (Lephart et al., 1995), testis (Valladares and Payne, 1979) and adipocytes (Simpson et al., 1989). Molecular cloning of aromatase cDNA revealed the existence of a single enzyme in most species including trout (Tanaka et al., 1992), chicken (McPhaul et al., 1988), rat (Hickey et al., 1990), mouse (Terashima et al., 1991), bovine (Hinshelwood et al., 1993) and human (Harada, 1988). The presence of aromatase activity in CNS has been suggested from biochemical studies demonstrating the androstenedione conversion into estrone in the rat brain (Naftolin et al., 1972, Roselli et al., 1985). Immunocytochemical studies have shown that aromatase is expressed in neurons but not in glial cells (Lephart, 1996). Aromatase expression has been also evidenced in the human temporal cortex (Yague et al., 2006).
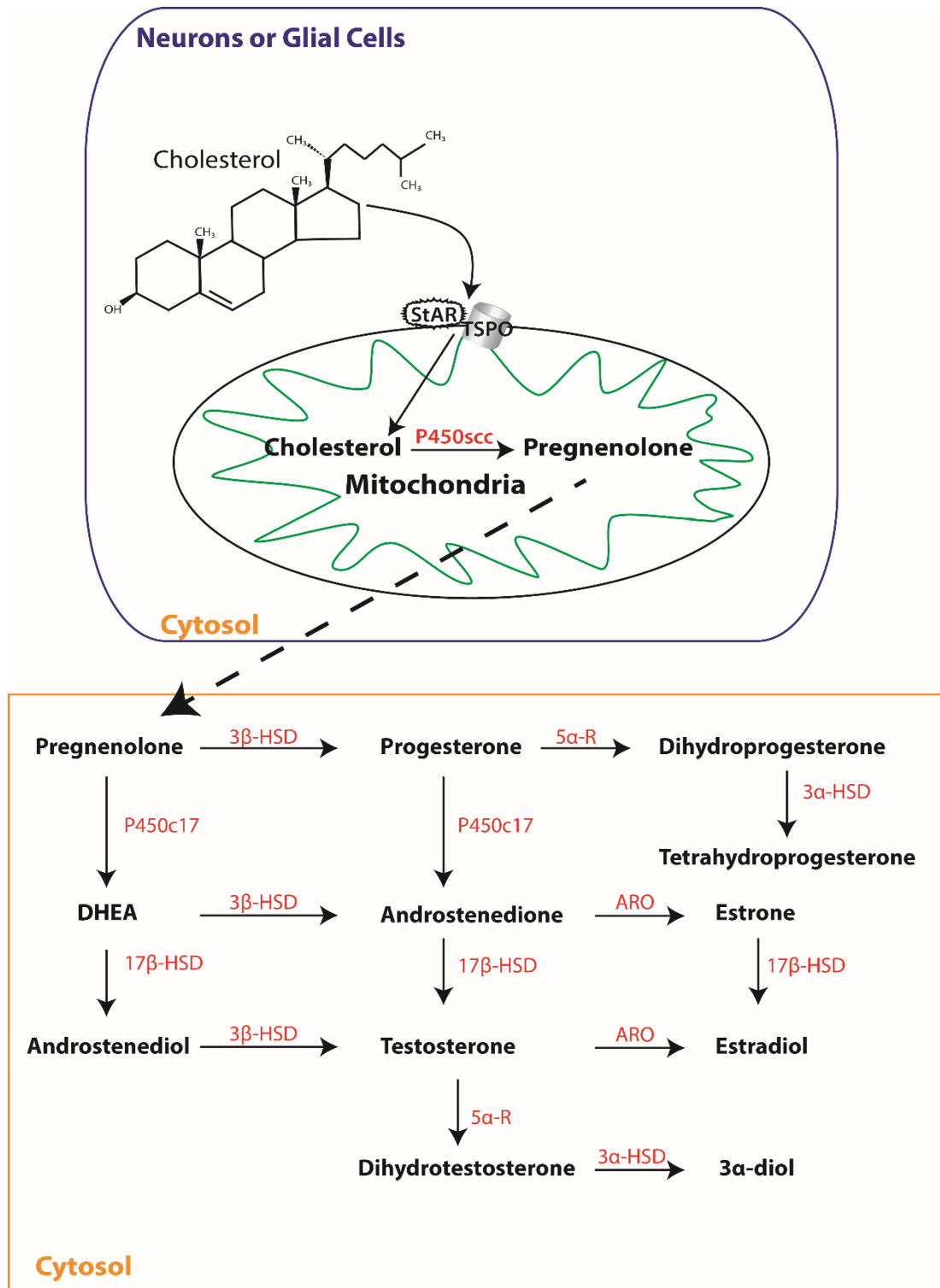
Figure 3. Steroidogenesis in neurons and glial cells. Schematic representation of neurosteroidogenesis. In red the enzymes involved in this process: Cytochrome P450 side chain cleavage (P450scc); 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450c17 (P450c17), 5α-reductase (5α-R), 17β-hydroxysteroid dehydrogenase (17β-HSD) and aromatase (ARO). The first step is the translocation of cholesterol from the cytosol to the inner mitochondrial membrane, via the 18 kDa translocation protein (TSPO) and the steroidogenic acute regulatory protein (StAR), where the conversion into pregnenolone by the P450scc occurs.

Neuroactive steroids, role in the central nervous system

Based on behavioral responses evoked in animals by synthetic steroid injections, several studies suggest that neuroactive steroids are involved in the control of various important neurophysiological processes.

Classical and non-classical receptors

As mentioned above, neuroactive steroids are actively metabolized by several enzymes such as 3β-HSD and 5α-R. These enzymatic steps exert an important role in the mechanism of action of neuroactive steroids, since active metabolites of these molecules exert their effects by a variety of mechanism, including the activation of classical steroid receptors signaling mediated by progesterone receptor (PR), androgen receptor (AR) and estrogen receptors (ERs), which are present in different isoforms, such as A and B or α and β (Melcangi et al., 2008). On the other hand, some of these metabolites may also exert their effects by binding to membrane receptors, such as GABA-A and GABA-B receptors, glutamate N-Methyl-D-aspartate (NMDA) receptor, AMPA and kainate subunits, membrane PROG receptors (mPRs) and pregnane X receptor (PXR) (Melcangi et al., 2008, Almey et al., 2014, Nag and Mokha, 2014, Schumacher et al., 2014, Qin et al., 2015). For instance, while DHP, like is precursor PROG, still interacts with PR, the further metabolites THP and isopregnanolone modulate the activity of GABA-A receptor. However, only THP is able to bind and activate the GABA-A receptor, while isopregnanolone antagonizes the THP effect (Melcangi et al., 2008). Similarly, DHT like its precursor T, interacts with AR, but the further metabolites of DHT, 3α-diol and 3β-diol, act by different mechanisms. Thus, 3α-diol is a GABA-A receptor agonist, while 3β-diol is an ERβ agonist (Handa et al., 2008, Melcangi et al., 2008).

On the other hand, it is important to remember that also neuroactive steroid substrates, such as DHEA and PROG have been recently reported to exert effects via non-classical steroid mechanisms. For instance, in case of DHEA, it is unclear whether it is able or not to interact with AR (Lu et al., 2003, Mo et al., 2004, 2006, 2009), but available observations indicate a role for GABA-A receptors in DHEA signaling (Maninger et al., 2009). Moreover, in case of PROG, also in the nervous system, like in other tissues, classical PR may move to the cytoplasm or the plasma membrane and interacts with components of intracellular signaling pathways, such as kinases. This is also the case of ERs (Schumacher et al., 2014). Furthermore, picomolar

concentrations of PREG sulfate are able to increase the intracellular response to glutamate at synaptic NMDA receptors via the phosphorylation of cAMP response element-binding protein (Smith et al., 2014).

Actions during development and adult life

The classical action of neuroactive steroids (i.e. which includes gonadal steroids and neurosteroids) in the nervous system is via the activation of nuclear receptors. However, these molecules are also able to activate different membrane or cytoplasmic signaling pathways. Therefore, hormonal action is the result of a combination of rapid signaling and transcriptional regulation (Nadal et al., 2001, Mannella and Brinton, 2006). Neuroactive steroids affect neural development and the function of the nervous system acting on specific moments during fetal, early postnatal or adult life, inducing different effects such as the generation of sexual dimorphism in the nervous system. For instance, in the early post-natal development, the main hormone implicated is testosterone, which is locally metabolized by neural tissue to DHT and estradiol by the 5α-R and aromatase respectively. DHT is a ligand of the AR while estradiol is a ligand of the ERs and are both involved in the organizational effects of testosterone in the nervous system. The organizational effects of testosterone and its metabolites generate male-specific traits in specific regions of the brain and spinal cord, resulting in differences in the morphology, size and number of neurons and glial cells, the density of neuronal and glial processes in the neuropil and the number of synapses between males and females (Cooke et al., 1998, Simerly, 2002). Neuroactive steroids may promote sex differences in synaptic connectivity by regulating microtubule assembly in neuronal processes, one of the key events involved in neurite elongation (Melcangi et al., 2008). Actions of gonadal steroids on glial cells may also be highly relevant for the sexual differentiation of neuronal connectivity (Garcia-Segura and Melcangi, 2006). Furthermore, glial cells express receptors for steroids and participate in steroid metabolism (Garcia-Segura and Melcangi, 2006). These cells also participate in the synthesis of endogenous steroids (i.e. neurosteroids) by the nervous system.

Neuroactive steroids have different effects in the adult CNS. They act on brain regions involved in the control of sex behavior and neuroendocrine regulation, modulating the release of neurotransmitter and the expression and function of neurotransmitter receptors and inducing plastic functional remodeling of synapses (Olmos et al., 1989, Csakvari et al., 2007) and associated glial processes (Garcia-Segura et al., 1994a, Garcia-Segura et al., 1994b). In

addition, cognitive brain regions are also affected by neuroactive steroids and these hormones regulate the number of dendritic spines and synapses and the induction of long-term potentiation in the CA1 region of the rat hippocampus (Woolley et al., 1990, Leranth et al., 2000). Neuroactive steroids also regulate adult neurogenesis in the dentate gyrus (Galea and McEwen, 1999, Galea et al., 2006). These plastic actions of neuroactive steroids in the hippocampus are associated to anti-depressive effects and the modulation of learning and memory processes (Parducz et al., 2006, Frye, 2007).

Role in neurological disorders and therapeutic use of neuroactive steroids

Pathological conditions modify neuroactive steroid levels in CNS. A specific and sensitive analytical technique, such as mass spectrometry, has been of great importance to determine the influences of pathological status on the levels of these molecules. Indeed, as demonstrated in several experimental models and clinical studies, neurodegenerative and psychiatric disorders affect neuroactive steroids levels (Weill-Engerer et al., 2002, Labombarda et al., 2006, Meffre et al., 2007, Caruso et al., 2008a, Mensah-Nyagan et al., 2008, Roglio et al., 2008, Caruso et al., 2010, Giatti et al., 2010, Pesaresi et al., 2010, Luchetti et al., 2011a, Noorbakhsh et al., 2011, Caruso et al., 2013a, Giatti et al., 2013, Melcangi et al., 2013, Caruso et al., 2015). For instance, in 3xTg-AD mice, an experimental model of Alzheimer's disease (AD), age-related gliosis and accumulation of β-amyloid protein were associated with modified levels of PROG, and T metabolites (Caruso et al., 2013a). Altered neuroactive steroid levels were also reported in post-mortem brain tissue of AD patients (Yue et al., 2005, Marx et al., 2006, Luchetti et al., 2011a, Luchetti et al., 2011b, Rosario et al., 2011).

In Parkinson's disease (PD) altered levels of neuroactive have been also reported. Indeed, a significant decrease in the levels of PREG and DHP as well as an increase of isopregnanolone levels were observed in the striatum of 6-hydroxydopamine (6-OHDA) injected rats (Melcangi et al., 2012). Moreover, DHP levels were also decreased in the cerebrospinal fluid (CSF) of PD patients. In agreement, the expression of steroidogenic enzymes is modified in the brain of PD patients. In particular, mRNA levels of the enzyme converting PROG into DHP and T into DHT (i.e. 5α-R) were decreased in the substantia nigra (di Michele et al., 2003).

Alterations in neuroactive steroid levels were also reported in multiple sclerosis (MS). Indeed, as recently observed in CSF of male adult patients affected by relapsing remitting (RR-MS), the levels of PREG and isopregnanolone were increased together with decreased levels of DHP and THP (Caruso et al., 2014). Moreover, an increase in 3α-diol and 17β-estradiol levels associated with a decrease in DHT levels also occurred. Interestingly, the alterations in neuroactive steroids depend on the forms and phases of MS. This feature was evident in experimental model of MS. For instance, decreased PROG levels, together with increased THP levels and decreased isopregnanolone levels have been detected in the spinal cord during the acute phase of experimental autoimmune encephalomyelitis (EAE) (Giatti et al., 2010), but not during the chronic phase (Caruso et al., 2010). Changes in the plasma and/or CSF levels of neuroactive steroids have been also reported to be associated with depression, anxiety, premenstrual dysphoric disorder, postpartum depression, post-traumatic stress, schizophrenia and impulsive aggression (Romeo et al., 1998, Uzunova et al., 1998, Marx et al., 2009, Rupprecht et al., 2010, Marx et al., 2011, Backstrom et al., 2014, Schule et al., 2014).

In the last few years, the finding that neuroactive steroids may be considered as neuroprotective agents has attracted the attention of several investigators. Indeed, it is now clear that neuroactive steroids, like progesterone and its derivatives (i.e. dihydroprogesterone and allopregnanolone) and dehydroepiandrosterone exert a variety of neuroprotective effects. For instance, progesterone metabolites including DHP and THP locally produced in the brain protect rat hippocampal neurons form kainic acid excitotoxicity in vivo (Ciriza et al., 2004, Griffin et al., 2004). Moreover, anti-inflammatory effects have been reported by in vivo observations. For instance, in EAE animals, estrogens as well as PROG and DHT are anti-inflammatory agents reducing pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and increasing anti-inflammatory molecules such as transforming growth factor-β2 and IL-6 (Giatti et al., 2012b, Spence and Voskuhl, 2012, Giatti et al., 2013, Giatti et al., 2015b). In experimental models of injury, such as the traumatic brain injury (TBI), the treatment with PROG reduces, in the cerebral cortex, edema, accumulation of astrocytes, nuclear factor kappa β p65, IL-1β and TNF-α (Garcia-Estrada et al., 1993, Grossman et al., 2004, He et al., 2004, Pettus et al., 2005, Feeser and Loria, 2011).

All these findings suggest that that neuroactive steroids may be candidates as new protective agents and therapeutic tools to counteract neurodegenerative events (Schumacher et al.,

2003, Veiga et al., 2004, Garcia-Ovejero et al., 2005). Moreover, some important aspects should be taken into consideration. For instance, it is important to highlight that the systemic treatment with neuroactive steroids may cause endocrine side effects. Indeed, steroid receptors are widely expressed in many tissues. Thus, an alternative may be the use of molecules that may activate steroid receptors in the CNS but not in the other tissues. However, as already mentioned, the actions of neuroactive steroids may depend on its active and fast conversion into metabolites. On this basis, for some neuroactive steroids the use of synthetic receptor ligands that cannot be metabolized in other active agents may be unsuccessful or evoke limited effects in comparison to what observed with the natural neuroactive steroids (Giatti et al., 2015a, 2015b).

Therefore, a promising therapeutic strategy may be represented by inducers of steroidogenesis, such as ligands of StAR and TSPO (Figure 4), which are also involved in the intramitochondrial transport of cholesterol. For instance, in the adrenal glands LXRs modulates StAR (Cummins and Mangelsdorf, 2006). Moreover, the activation of LXRs, inducing genes involved in cholesterol efflux, like the ATP-binding cassette family of transporters (i.e. ABCA1 and ABGC1), promotes cholesterol utilization (Cummins and Mangelsdorf, 2006). Recently, Mitro et al., (2012) reported that the activation of LXRs in an experimental model of diabetes, is able to increase neuroactive steroid levels directly in the central nervous system without altering the plasma levels of steroids (Mitro et al., 2012).
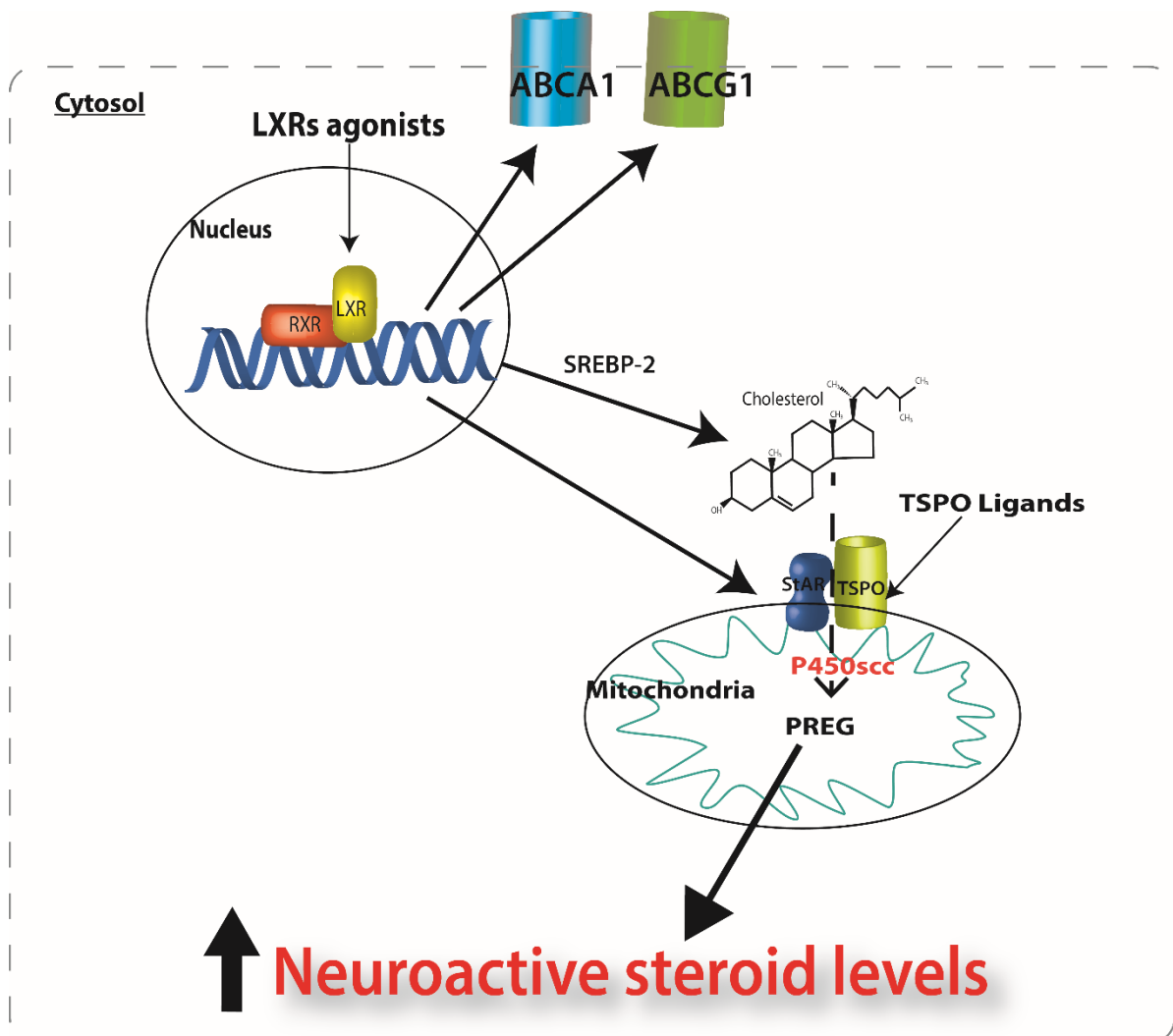
Figure 4. Promotion of neurosteroidogenesis. This schematic figure represents the possible strategy to increase the levels of neuroactive steroids using synthetic agonists of the Liver X receptors (LXRs), that may increase the synthesis of cholesterol by the promotion of Sterol regulatory element-binding protein 2 (SREB-2). In addition, the promotion of the mobilization by the ATP-binding cassette (ABC) A1 and A2 may increase neuroactive steroids levels. Another strategy is the stimulation of the 18 kDa translocation protein (TSPO) by specific ligands that promote the influx of cholesterol into the mitochondrial compartment.

Cholesterol: regulation and role in the nervous system

Cholesterol is an amphipathic lipid molecule synthesized by all animals. It is the major sterol in animal tissues and an essential structural component of the cell membranes. Indeed, it is required to maintain the structural integrity, fluidity, and maintenance of lipid rafts (Simons and Ikonen, 2000, Zhang et al., 2009). In addition, cholesterol serves as a precursor for a variety of products with specific biological activities such as steroid hormones (i.e. produced after cholesterol conversion into PREG) (Nelson, 2004).

In the central nervous system cholesterol is an essential component of synapses which, aside from directly enhancing presynaptic differentiation, enables continuous synaptogenesis and provides stability of evoked transmitter release (Goritz et al., 2005). Moreover, it is involved in the maintenance of several neuronal functions such as the conduction of the action potential, the stabilization of synapses, and the formation of lipid rafts (i.e. centers of signaling molecules regulating neurotransmission) (Block et al., 2010). Cholesterol is ubiquitously distributed throughout the brain where it is produced in situ (Dietschy and Turley, 2004) and the maintenance of steady levels of cholesterol is crucial for proper CNS functioning (Smiljanic et al., 2014). There is no evidence for a net transfer of cholesterol from the bloodstream into the CNS or the spinal cord, probably because lipoproteins, which are responsible for intercellular transport of sterol and other lipids, are not able to cross the blood brain barrier (BBB) (Dietschy and Turley, 2004). Although the CNS constitutes 2% of the total body weight, it contains five to ten times more cholesterol than any other organ, which corresponds to the 25% of the total sterol present in whole body pool (Pfrieger, 2003, Dietschy and Turley, 2004). In particular, the greatest amount of unesterified cholesterol is contained in myelin membrane (Segatto et al., 2012).

Cholesterol homeostasis is accomplished through a sophisticated regulation of synthesis, transport and elimination of excessive cholesterol from the brain (Smiljanic et al., 2014).

Biosynthesis of cholesterol

Cholesterol synthesis is very high in the developing brain, thus reflecting the synthesis of a large quantity of cholesterol-rich myelin membrane. However, this rate declines at low and constant levels during adulthood (Dietschy and Turley, 2004). Cholesterol biosynthesis could be differently modulated in brain regions of adult male rat. In parallel, differences in the modulation of the proteins involved in intracellular cholesterol homeostasis control occur (Segatto et al., 2012). Indeed, several studies have demonstrated that proteins involved in the biosynthesis (Keller et al., 1985, Segatto et al., 2011) and trafficking of cholesterol (Segatto et al., 2012) exhibit a specific brain region pattern of activation state and protein expression.

As previously reported, brain cholesterol is predominantly derived from de novo synthesis (Bjorkhem et al., 1998) and requires multiple regulatory mechanisms involving synthesis, transport and metabolism to maintain physiological levels. Biosynthesis of cholesterol is a multistep energetic-consuming pathway (Figure 5) comprising multiple intermediates and mediating enzymes (DeBose-Boyd, 2008) starting with acetyl coenzyme A, which is processed to desmosterol or lathosterol, respectively the direct precursor in the Bloch pathway and in the Kandutsch-Russel pathway (Brown et al., 1980). These two pathways are alternate during the cerebral life, indeed it has been reported that the Bloch pathway (i.e. which use the desmosterol as precursor of cholesterol) is preferred in the young life of rodents, while the Kandutsch-Russel pathway prevails in older rodents (Lutjohann et al., 2002, Smiljanic et al., 2013).

SREBP-2 and HMG-CoA Reductase, the rate limiting step

Cholesterol synthesis is a highly regulated process controlled by the master transcriptional regulator sterol regulatory element-binding protein 2 (SREBP-2). The gene expression of this protein is regulated by insulin and through feedback regulation by sterols, including cholesterol itself (DeBose-Boyd, 2008). SREBP-2 is transcribed and translated into an inactive precursor that is sequestered in the endoplasmic reticulum (ER).

However, when sterol levels are low, the sterol sensor SREBP cleavage-activating protein (SCAP) is able to chaperone SREBP-2 to the Golgi apparatus where it is cleaved to release a transcriptionally active form that can enter the nucleus (Brown and Goldstein, 2009). SREBP-2, in the nucleus, binds the sterol-responsive elements (SREs) containing promoters on target

genes, including those encoding cholesterol biosynthetic enzymes such as 3-hydroxy-3methylglutaryl coenzyme-A (HMG-CoA) synthase and reductase (Wang et al., 2002, Tarr and Edwards, 2008). In addition, recent findings have reported the presence of SREBP-2 in the cerebral cortex and hippocampus of the brain (Ong et al., 2000, Kim and Ong, 2009). Moreover, HMG-CoA reductase, the rate-limiting step enzyme in the biosynthetic way, is also highly expressed in the neurons of these brain areas (Korade et al., 2007). This enzyme is responsible of the 3-hydroxy-3methylglutaryl coenzyme-A conversion into mevalonate occurring in the ER (Siperstein and Fagan, 1966, Ong et al., 2010, Rozman and Monostory, 2010, Suzuki et al., 2010, Trapani et al., 2011, Segatto et al., 2012, Segatto et al., 2013).

DHCR24, the sterol synthesis rate

The second part of the sterol synthesis pathway begins with the first committed step in sterol synthesis that is catalyzed by the farnesyl-diphosphate farnesyltransferase and results in the formation of squalene. Epoxidation and cyclization of squalene leads to the first sterol intermediate lanosterol. The conversion of lanosterol to cholesterol comprises a series of demethylations, double bond reductions and desaturations. The order of reactions can differ resulting in distinct cholesterol precursor. As previously mentioned the immediate precursor of cholesterol in the Bloch pathway (Figure 5) is desmosterol (24-dehydrocholesterol, 24DHC) which is converted to cholesterol by 24-dehydrocholesterol reductase (DHCR24), while 7-dehydrocholesterol (7DHC) that is converted to cholesterol by 7-dehydrocholesterol reductase (DHCR7) is the final step in the Kandutsch-Russell pathway (Saher and Stumpf, 2015).
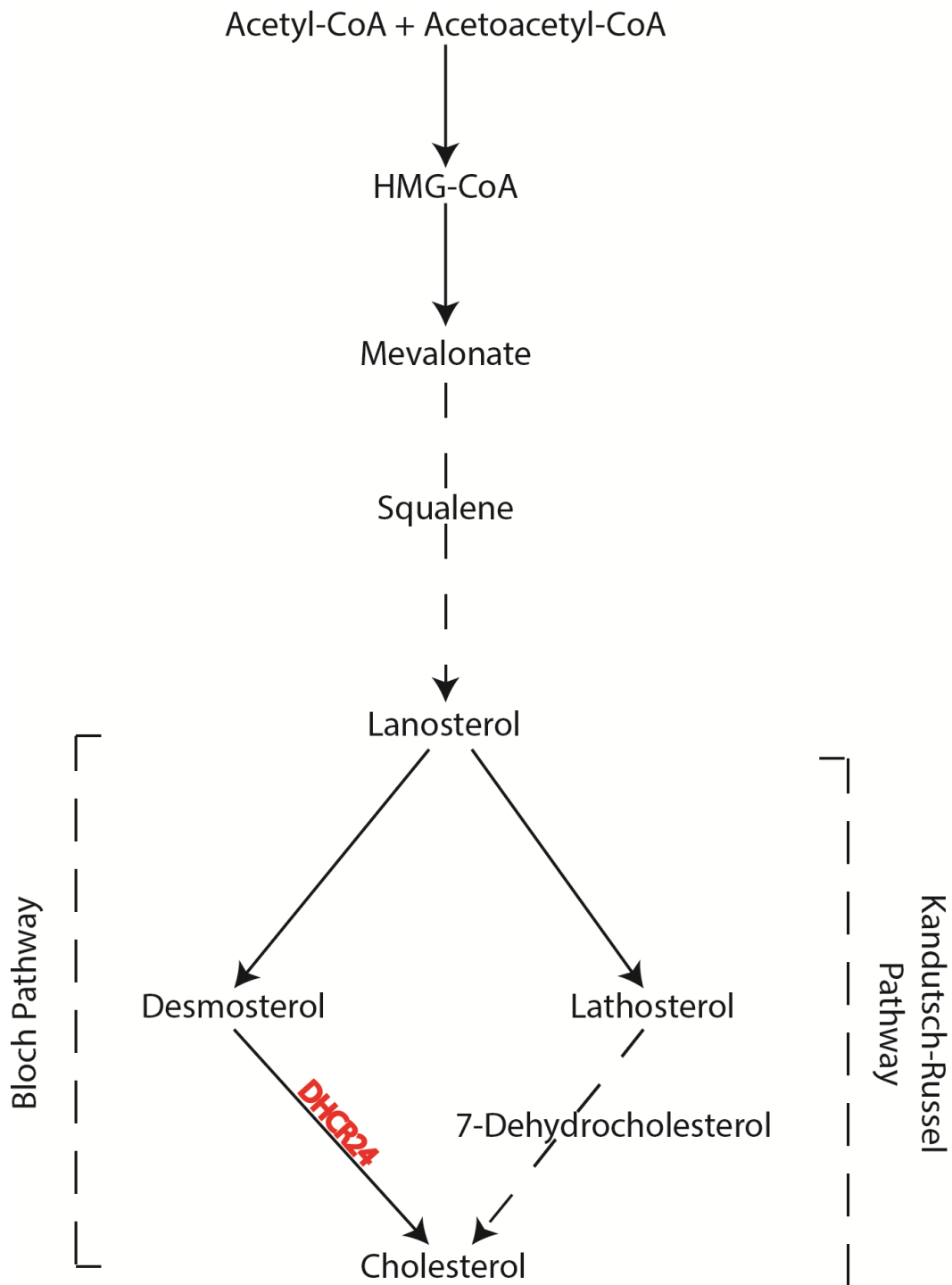
Figure 5. Biosynthetic pathways of cholesterol. This figure represents the two possible biosynthetic pathways of cholesterol. Biosynthesis of cholesterol is a multistep energetic-consuming pathway comprising multiple intermediates and mediating enzymes starting with acetyl coenzyme A, which is processed to desmosterol or lathosterol, respectively the direct precursor in the Bloch pathway and in the Kandutsch-Russel pathway. These two pathways are alternate during the cerebral life, indeed it has been reported that the Bloch pathway is preferred in the young life of rodents, while the Kandutsch-Russel pathway prevails in older rodents

DHCR24 not only plays an essential role in the regulation of cholesterol synthesis, but it is also important in other cellular processes, such as signaling, the formation of lipid rafts, mediating cell stress responses and regulating steroidogenesis. Indeed, some studies have demonstrated that desmosterol, like cholesterol, can also serve as a precursor for steroidogenesis (Goodman et al., 1962a, b), with similar (Goodman et al., 1962b) or better (Arthur et al., 1976) efficiency than cholesterol. Therefore, if cholesterol synthesis is shut down at DHCR24, synthesis of steroid hormones should continue unhindered. DHCR24 is finely regulated through feedback systems that operate at transcriptional and post-translational levels. Although this feedback is mainly through sterols, the hormones, the growth factors signals, and epigenetic signals may also affect regulation. SREBPs transcription factors are major regulators of lipid homeostasis. Three isoforms of SREBP exist in mammals, which activate specific sets of genes. For instance, SREBP-2 activates genes such as HMG-CoA reductase (i.e. first step in the cholesterol synthesis) (Vallett et al., 1996), and low density lipoprotein receptor (i.e. the receptor involved in the uptake of cholesterol) (Briggs et al., 1993). Low sterol levels are sensed by SCAP, which actives SREBPs by proteolytic cleavage in the Golgi. This active factor enters into the nucleus to bind to SREs in target genes, and thereby increase cellular lipid levels. Increased flux through the mevalonate pathway creates sterol and oxysterol by-products, which, like cholesterol, can act as signals to reduce cholesterol synthesis (Gill et al., 2008, Bjorkhem, 2009) by inhibiting SREBP activation and by acting as ligands for the nuclear receptor, liver X receptor (LXR). This mechanism activates LXR and induces expression of genes involved in sterol efflux (e.g. ABCA1) through binding to LXR response element within their promoters (Zelcer and Tontonoz, 2006). Thereby, the homeostasis is maintained through the reduction of sterol levels. One study reported that DHCR24 is a target gene of LXR (Wang et al., 2008), and many studies have supported a link between SREBP activity and DHCR24 expression (Bae and Paik, 1997, Horton et al., 2003, Demoulin et al., 2004, Kallin et al., 2007, Ramos et al., 2012). Since SREBP and LXR have opposing effects on cellular sterol levels, it would be paradoxical if DHCR24 were regulated by both transcription factors. Indeed, Zerenturk et al. (2012) have reported, in a Chinese hamster ovary (CHO)-7 cell line, that LXR does not influence DHCR24 expression while SREBP-2 plays the major role in sterol-regulated expression of DHCR24 (Zerenturk et al., 2012). Like other cholesterogenic genes, DHCR24 expression is very low in the absence of SREBP-2 (Zerenturk et al., 2012). Furthermore, sex steroids are also regulators of DHCR24. For instance, androgens are positive regulators

increasing gene expression via activation of the AR receptors (Nelson et al., 2002, Benvenuti et al., 2005, Bonaccorsi et al., 2008, Luciani et al., 2008, Zu et al., 2012). It was proposed that androgens indirectly affect DHCR24 expression by up-regulation of SCAP, promoting SREBP-2 activation (Heemers et al., 2006). Moreover, PROG, the major gestational steroid hormone, can inhibit DHCR24 activity. For instance, in cultured cells, PROG and other similar progestins inhibit cholesterol synthesis, accompanied by accumulation of desmosterol (Metherall et al., 1996, Thewke et al., 1998, Lindenthal et al., 2001). Finally, glucose metabolism is also involved in DHCR24 regulation, Giannini et al. (2008), inferring that DHCR24 is involved in diabetic neuropathy, since IGF-1 stimulates and low glucose inhibits DHCR24 expression in neuronal cells.

Up to 30% of total sterols and 80% of the newly synthesized sterols, desmosterol is the most common precursor of cholesterol in the brain of newborn mice (Jurevics and Morell, 1995, Fliesler et al., 1999, Keller et al., 2004, Jansen et al., 2012). Temporal cholesterol precursor accumulation indicates high sterol synthesis rates. Desmosterol rather reflects sterol synthesis in newly differentiated astrocytes than oligodendrocytes, a hypothesis that is supported by data in cultured astrocytes (Mutka et al., 2004, Nieweg et al., 2009). For instance, in DHCR24 null mice, the severity of defects could be correlated directly to the level of remaining cholesterol. These studies imply that at least minimal amounts of cholesterol are essential to maintain cell function. Disorders caused by mutations in the sterol biosynthesis pathway cause, in addition to other severe developmental malformations, deficits in myelination underlining the importance of cholesterol for myelin membranes (Waterham, 2006, Kanungo et al., 2013).

As previously mentioned, cholesterol is necessary for the normal function and to maintain the morphology of the CNS. The function of neuronal cell is impaired not only due to lack but also to the surplus of cholesterol (Ko et al., 2005, Pooler et al., 2006). Defects of cholesterol homeostasis in the adult brain are linked to neurodegenerative diseases like Niemann-Pick Type-C disease or Alzheimer's disease (Block et al., 2010, Madra and Sturley, 2010, Di Paolo and Kim, 2011, Wang et al., 2011). It is well known that neuronal cells regulate their cholesterol content by fine feedback mechanisms balancing biosynthesis, metabolism, and trafficking. As previously mentioned, intracellular levels of sterols can be detected by SREPBs. These regulators are membrane-bound transcription factors modulating the expression

enzymes of cholesterol and fatty acid biosynthesis as well as lipoprotein receptors (Brown and Goldstein, 1999). SREBPs can either increase cholesterol synthesis and uptake (in sterol-dependent cells) or decrease cholesterol-synthesizing enzymes when sterols are overload in cells (DeBose-Boyd et al., 1999, Nohturfft et al., 2000).

Metabolism of cholesterol

ACAT-1/SOAT-1 and HSL, the storage and bioavailability

The homeostasis of cholesterol is one of the most intensely regulated processes in biology. For instance, the excess of unesterified or free cholesterol is esterified into cholesteryl esters (CEs) and stored in cytoplasm lipid droplets (Figure 6). In the brain, this intracellular pool of cholesterol is involved in the synaptic and dendritic formation and in the membrane remodeling (Dietschy and Turley, 2004). The equilibrium between free and esterified cholesterol is controlled by acyl-coenzyme A: cholesterol acyltransferase (ACAT) also known as sterol O-acetyltransferase (SOAT), an integral membrane protein localized in the endoplasmic reticulum (ER) (Chang et al., 2009). Two isoforms of this enzyme, ACAT-1 and ACAT-2, have been identified in neural and non-neural tissues (Rudel et al., 2001). ACAT-1 is the isoform present in the brain structures (Lee et al., 1998). ACAT also acts as cholesterol sensor, and its expression is induced by increased cholesterol concentrations (Brown and Jessup, 2009). ACAT-1 activity is coordinately increased to maintain the level of free cholesterol within the cell in a fairly narrow range (Brown and Goldstein, 1986).

Cholesteryl esters are formed by cholesterol and fatty acyl coenzyme A such as arachidonoyl-coenzyme A, and hydrolyzed back to cholesterol continuously, forming the cholesterol/cholesteryl ester cycle (Brown et al., 1980). Hydrolysis of cholesteryl esters provides cholesterol and fatty acids for formation and maintenance of the membrane, lipoprotein trafficking, lipid detoxification, evaporation barriers, and fuel in times of stress or nutrient deprivation (Turkish and Sturley, 2009). Hormone-sensitive lipase (HSL) is able to hydrolyze cholesteryl esters into free cholesterol and fatty acids. HSL is a multifunctional enzyme playing an important role in a number of physiological processes (Osuga et al., 2000, Ascoli et al., 2002, Yeaman, 2004, Manna et al., 2009, Feingold et al., 2012). Notably, HSL is the primary neutral cholesteryl esters hydrolase (NCEH) in the steroidogenic tissues, and disruption of HSL gene in mice results in a marked attenuation of NCEH activity in the adrenal

and testes accompanied with profound morphological alterations in these tissues. For instance, male mice homozygous for the mutant HSL allele were sterile (Osuga et al., 2000) while HSL null male mice exhibit several testicular abnormalities, including decreased weight, vacuolated seminiferous tubules, reduced spermatids, and sterility (Osuga et al., 2000, Li et al., 2002a, Wang et al., 2004b). Hormonal control of HSL activity is chiefly mediated by phosphorylation of several serine residues, by cyclic AMP (cAMP)-dependent protein kinase A (PKA) as well as by other kinases (Osterlund, 2001, Krintel et al., 2009, Manna et al., 2009). Moreover, the activation of cAMP/PKA signaling enhances phosphorylation of HSL with its increased hydrolytic activity, and these events are tightly connected with StAR expression and steroid biosynthesis in gonad and adrenal cells (Ascoli et al., 2002, Manna et al., 2009). In keeping with this, deficiency of HSL affects StAR and steroid levels, demonstrating that HSL plays a vital role in regulating the steroidogenic response.
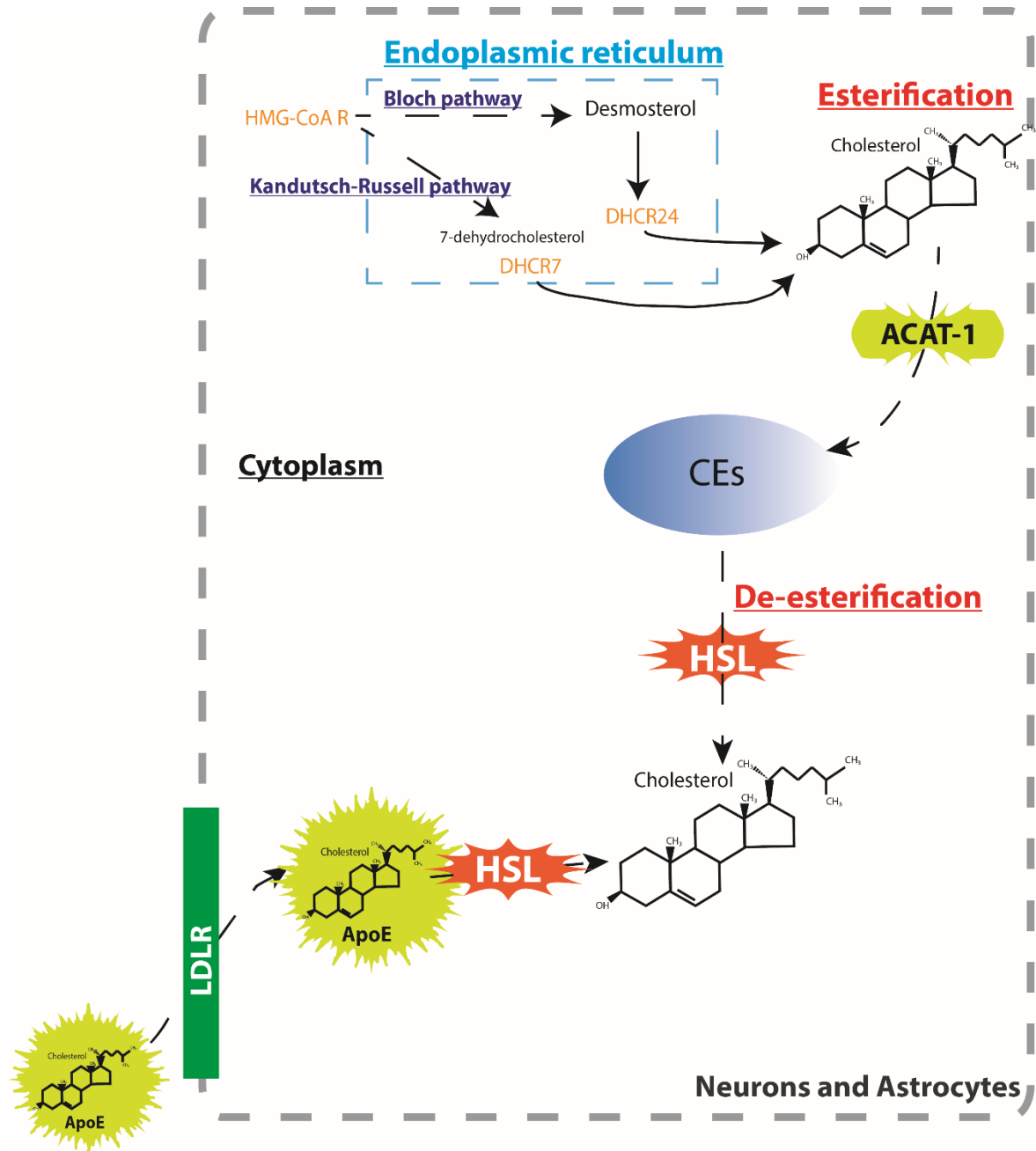
Figure 6. Storage and liberation of cholesterol. 3-hydroxy-3-methylglutaryl-Coa reductase (HMG-CoA R); 24-dehydrocholesterol reductase (DHCR24); 7-dehydrocholesterol reductase (DHCR7); Apolipoprotein E (ApoE); Low density lipoprotein receptor (LDLR); Sterol-o-acyl transferase (SOAT-1); Hormone sensitive lipase (HSL); Cholesteryl esters or esters of cholesterol (CEs). This diagram represents the modification that occur to the new-synthesized cholesterol and the cholesterol derive from the ApoE. Free cholesterol is toxic for the cell, for this reason the excess may be esterified by SOAT-1. When it becomes necessary HSL hydrolyze CEs to free cholesterol. Moreover, HSL can also hydrolyze the cholesterol contained into the ApoE.

Oxysterols, the metabolites and the oxidation state

When cholesterol reaches the maximum required level, is converted by neurons into the more polar derivatives called oxysterols. This class of molecules can be eliminated in the presence of HDL as lipid acceptor, and protects neurons from the toxic effect of cholesterol and their metabolites (Matsuda et al., 2013). Oxysterols are oxygenating cholesterol derivatives that are produced from enzymatic or non-enzymatic oxidation of cholesterol (Figure 7) (Smith et al., 1981, Addis, 1986). The introduction of an oxygen atom in cholesterol drastically reduces its half-life and directs the molecule to the excretion. Degradation and elimination are facilitated by the physical properties of oxysterols. These molecules are able to pass lipophilic membranes much more quickly than does cholesterol itself (Lange et al., 1995). The most obvious role of oxysterols is that they are precursors of bile acids. Moreover, another well-established role is that they represent a transport form of cholesterol, allowing elimination over biological membranes not dependent on specific transport proteins. In addition, oxysterols are important regulatory molecules under in vivo conditions. Indeed, it is well documented that side-chain oxidized oxysterols are efficient ligands and activators of the nuclear receptors LXRs (Lehmann et al., 1997). A knockout of these receptors leads to specific effects on cholesterol homeostasis. According to current concepts, oxysterols are the physiological activators of the receptors above mentioned (Lehmann et al., 1997, Bjorkhem, 2002). Oxidation of the sterol ring, in the cholesterol molecule, is almost always non-enzymatic, and leads compounds like 7-ketosterol (7-K), 7β-hydroxycholesterol (7β-OH) and 7α-hydroxycholesterol (7α-OH). However, 7α-OH may be also produced via an enzymatic mechanism by an enzyme of the superfamily of cytochrome P450 (e.g. CYP7A1). It was reported by Iuliano et al. (2015) that reactive oxygen species (ROS) are implicated in the formation of non-enzymatic oxysterols due to the elimination from the brain. As previously mentioned, oxysterols such as 24(S)-hydroxycholesterol (24(S)-OH), 25-hydroxycholesterol (25-OH) and 27-hydroxycholesterol (27-OH), can be produced by specific enzymatic reactions (Sottero et al., 2009, Iuliano, 2011).
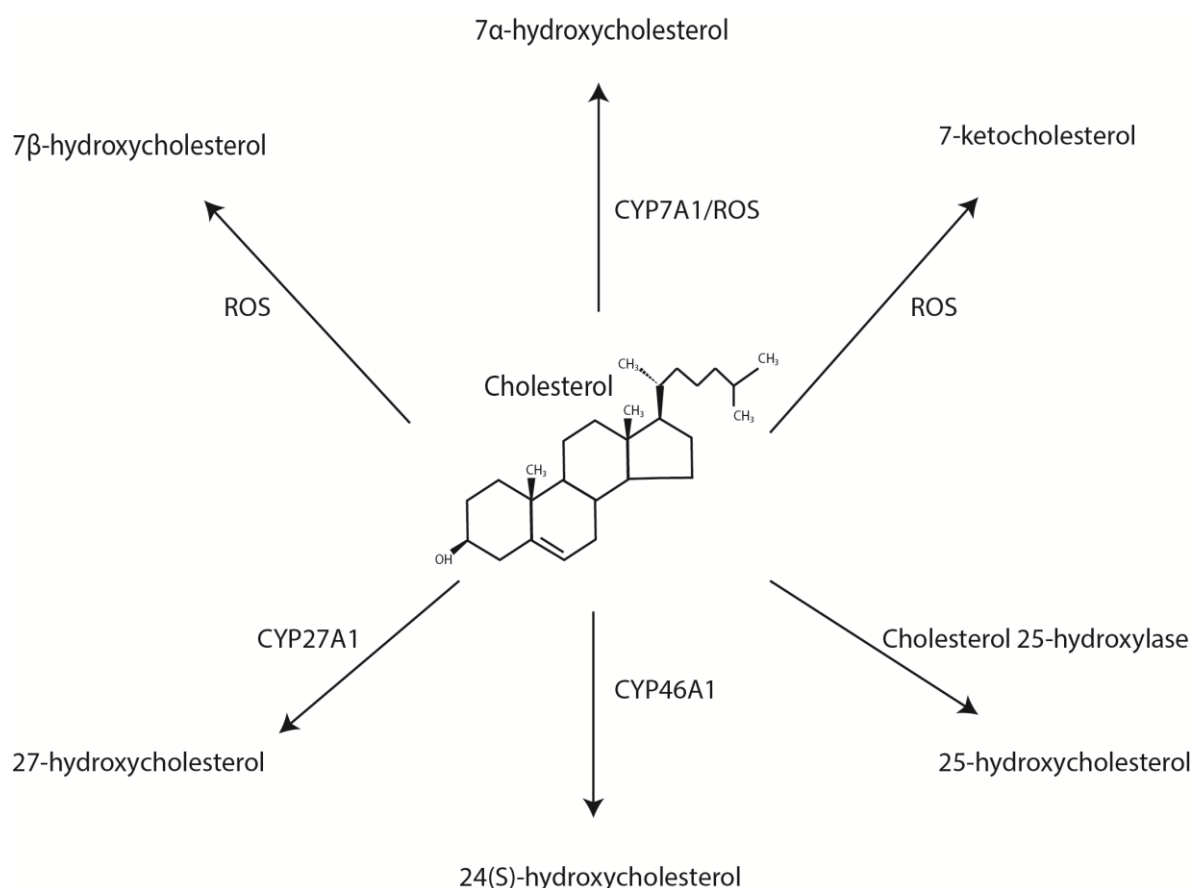
7α-hydroxycholesterol

7β-hydroxycholesterol

7-ketocholesterol

CYP7A1/ROS

ROS

ROS

Cholesterol

CYP27A1

CYP46A1

Cholesterol 25-hydroxylase

27-hydroxycholesterol

25-hydroxycholesterol

24(S)-hydroxycholesterol

Figure 7. Enzymatic and non-enzymatic oxysterols. Reactive Oxygen Species (ROS); Cytochrome 27-hydroxylase (CYP27A1); Cytochrome 24-hydroxylase (CYP46A1); Cytochrome 7α-hydroxylase (CYP7A1). Enzymatic cholesterol oxygenation reactions are mediated by different cytochrome P450 except for the cholesterol 25-hydroxylase, that is a non-heme iron protein. Non-enzymatic cholesterol oxygenation occurs in the presence of ROS

In particular, 24(S)-OH, which is actively converted by cholesterol 24(S)-hydroxylase (i.e. encoded by CYP46A1) is highly expressed in neurons and subsequently eliminated from brain tissue (Bjorkhem et al., 1997). Indeed, unlike cholesterol, this oxysterol can pass the BBB. In humans, the resulting flux of 24(S)-OH from the brain into the circulation is continuous and similar in magnitude to hepatic uptake of this species, indicating that most of the 24(S)-OH present in the circulation originates from the brain (Figure 8). Under normal conditions cholesterol 24-hydroxylase is mainly present in neuronal cells of the cerebral cortex, hippocampus, dentate girus, amygdala, putamen and thalamus (Leoni et al., 2013). In rat brains, the rate of 24(S)-OH synthesis is matched by the rate of synthesis of cholesterol, suggesting that there is a balance between synthesis and metabolism of cholesterol (Bjorkhem et al., 1997). Indeed, disruption of the CYP46A1 gene, in a mouse model, reduces the synthesis of new cholesterol in the brain by 40%. This data indicates that the new-genesis of cholesterol in the brain is depending also on the cholesterol turnover (Lund et al., 2003).
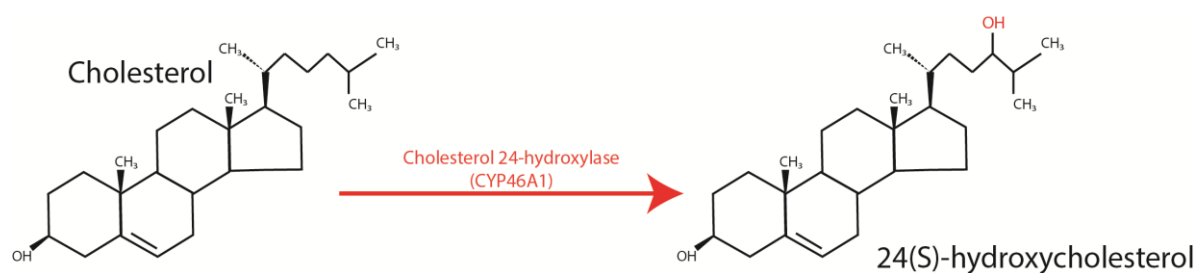
Figure 8. 24-hdyroxycholesterol, the main metabolite in brain. Example of cholesterol oxygenation. Cholesterol does not pass readily across the blood-brain barrier, but cholesterol produced in the brain can be oxidized by CYP46A1 to form the more soluble species 24(S)-hydroxycholesterol.

Oxysterols have a regulatory importance under in vivo conditions. For instance, these molecules influence the lipid synthesis mechanisms by acting on SRE and SREBP. These transcription factors regulate lipid homeostasis in vertebrate cells by activation of more than 30 genes involved in the synthesis and uptake of cholesterol, fatty acids, triglycerides and phospholipids as well as NADPH (Horton et al., 2002). Moreover, it is well documented that side-chain oxidized oxysterols are efficient ligands and activators of the LXRs that are regulators of cholesterol metabolism and seem to be regulators of the human cholesteryl ester transfer protein (Bjorkhem, 2002, Bjorkhem and Diczfalusy, 2002, Meaney et al., 2002). In particular, LXRα strongly modulates the brain cholesterol transporters (i.e. ABCA1, ABCG1, and ApoE) and CYP7A1 (encoding for 7α-hydrolase), which produces 7α-hydroxycholesterol (Chiang et al., 2001). Oxysterols bind LXRs with high affinity and are believed to be most important physiological activators for these receptors. However, it is not known if the oxysterols are ligands for these receptors also under in vivo conditions. Indeed, it is difficult to exclude a role for unmodified cholesterol, as either a ligand or an antagonist of these receptors (Bjorkhem, 2002). Finally, oxysterols may alter the synthesis of cholesterol. Indeed, in vivo studies performed by Saucier et al. (Saucier et al., 1989) have shown that several oxysterols present in mouse liver after cholesterol feeding are able to downregulate HMG-CoA reductase as well as block endogenous cholesterol synthesis. Similar results were obtained in astrocytes, where 24(S)-OH decreases the expression of HMG-CoA reductase and increases LXR regulated Apolipoprotein E (ApoE) expression (Horton et al., 2002).

Trafficking of cholesterol

ApoE, the carrier

ApoE is a major apolipoprotein in the CNS. After liver, the brain is the organ with the highest ApoE expression (Linton et al., 1991). The major function of ApoE is to participate in cholesterol homeostasis. Transcripts for ApoE are distributed throughout all regions of the brain and have been localized in astrocytes and microglia by in situ hybridization (Herz and Beffert, 2000). Indeed, primary cultures of hippocampal neurons from rat embryos and pro-simians have the capacity to internalize ApoE-containing lipoproteins secreted by glial cells through the members of LDL receptor (LDLR). Astrocytes are the major source of ApoE followed by oligodendrocytes, microglia, and ependymal layer cells (Mahley et al., 2006). Neurons may express ApoE under certain condition such as excitotoxicity injury (Xu et al., 1999). There is a dynamic exchange of cholesterol between neuronal and non-neuronal cells. In this contest, ApoE is the major transport protein for extracellular cholesterol and other lipids in the CNS (Lahiri, 2004).

The ApoE level is decreased in ABCA1 (i.e., a gene necessary for the ApoE lipidation) knockout mice (Wahrle et al., 2004, Hirsch-Reinshagen et al., 2005). Association with lipids is required for the stability of ApoE in the brain. Indeed, ApoE associated with lipid may induce a strong anti-apoptotic effect and protect cells against neurodegeneration through an intercellular signaling pathway (Liu et al., 2010).

LDLR, the uptake receptor

Numerous lipoprotein receptors of LDLR family have been identified in CNS (Herz, 2009, Pottier et al., 2012). Ligands for these receptors are ApoE-containing lipoproteins, lipids and other macromolecules (Ignatius et al., 1987, Pitas et al., 1987). Among them, the LDLR is the mainly expressed for the uptake of ApoE-containing lipoprotein particles in the brain. The uptake of molecules such as LDL and ApoE containing lipoproteins occurs through the LDLR, and it is a classic example of receptor-mediated endocytosis (Brown and Goldstein, 1976).

The LDLR binds the LDL/ApoE transporter and then is endocytosed by clathrin-coated vesicles and transported to acidic endocytic compartments, where cholesterol esters are hydrolyzed by acid lipase (Brown and Goldstein, 1986). After that, the LDLR dissociates from the

lipoprotein in the early endosome (due to the lower pH) and it is recycled back to the plasma membrane by vesicular mechanisms (Holtta-Vuori et al., 2002, Linder et al., 2007).

LDL receptor has high affinity for the ApoE lipoprotein particles secreted by glial cells (Fagan et al., 1996). The conformation and lipidation status of ApoE may affect the specificity of its receptor binding (Zhang and Liu, 2015). Indeed, poorly lipidated ApoE are more rapidly cleared in CSF (Hirsch-Reinshagen et al., 2004).

ABC transporters, more than efflux molecules

CNS expresses ATP-binding cassette (ABC) transporters (Kim et al., 2008), which are involved in many processes such as the regulation of lipid transport, as well as sterol efflux at the plasma membrane. It is now clear that tight regulation of brain cholesterol homeostasis is crucial for neurological function and that the alteration of cholesterol equilibrium can contribute to neurodegeneration (Dietschy and Turley, 2001, Puglielli et al., 2003). ABC transporters are essential component mediating lipid transport in CNS (Tachikawa et al., 2005). The critical classes of ABC transporters mediating homeostasis of cholesterol and lipid in the brain are ABCA1 and ABCG1 (Schmitz et al., 2000, Dean et al., 2001, Puglielli et al., 2003).

ABCA1 is expressed by neurons in embryonic and adult rodents (Wellington et al., 2002, Koldamova et al., 2003), and their levels are much higher than those occurring in astrocytes (Tarr and Edwards, 2008). Cholesterol is directly released bound to ApoE-containing lipoproteins that are present in CSF (Panzenboeck et al., 2002, Gosselet et al., 2009). ABCA1 catalyzes the initial transfer of lipids into lipid-free apolipoproteins, including ApoE, to form nascent particles, which are then fully lipidated in the second phase of efflux mediated by ABCG1 (Gelissen et al., 2006, Vaughan and Oram, 2006). Neuronal- and glial-specific ABCA1 deficiency leads to poor lipidation of ApoE, significant decrease of cholesterol level, and decreased level of ApoE in CNS. Cholesterol efflux in cultured astrocytes may be enhanced by agonists of LXRs. These nuclear receptors control the protein expression of ApoE, that are involved in the mediation of cellular cholesterol trafficking (Minagawa et al., 2009).

ABCG1 is highly expressed in neurons, in particular, is abundant in the hippocampus, where it was found in CA1, CA2, CA3 as well as in the dentate gyrus (Tansley et al., 2007). One of the features differentiating ABCA1- and ABCG1-mediated cholesterol efflux is the nature of the extracellular cholesterol acceptor. Several studies have shown that ABCA1 preferentially

stimulates cholesterol efflux to lipid free acceptors, such as ApoE whereas ABCG1 is more selective for lipidated lipoprotein complexes, such as HDL (relevant for peripheral reverse cholesterol transport) and lipidated ApoE (relevant in CNS) (Nakamura et al., 2004, Wang et al., 2004a, Kennedy et al., 2005, Kim et al., 2007). In CNS, a synergistic process relying on initial ABCA1-dependent efflux of phospholipids to lipid free acceptors (which may occur concomitantly with cellular apolipoprotein secretion) is apparently followed by ABCG1-dpendent enrichment of the lipoprotein complexes with cholesterol (Gelissen et al., 2006, Karten et al., 2006, Vaughan and Oram, 2006). In mice model overexpressing ABCG1, cholesterol intermediates (e.g. lathosterol, lanosterol and desmosterol) were all significantly reduced. On the contrary, ABCG1 deficient mice, displayed complementary increases in these cholesterol intermediates in a gene dose-responsive manner. However, the total cholesterol levels were unchanged by ABCG1 overexpressing or deficiency. Moreover, 24(S)-OH was significantly reduced by 15% in mice overexpressing ABCG1 compared with controls, and conversely increased by 20% in ABCG1 deficient mice. No differences were found for other oxysterols such as 27-OH (Burgess et al., 2008).

Summarizing, the homeostasis of cholesterol in the CNS is composed by synergic actions of storage, metabolism, and trafficking (or efflux). In particular, neurons and glial cells may handle excess cholesterol by esterification and subsequent intracellular storage, or by direct excretion via ABC transporters and/or by conversion to enzymatic oxysterols such as 24(S)-OH, 25-OH and 27-OH (Figure 9).
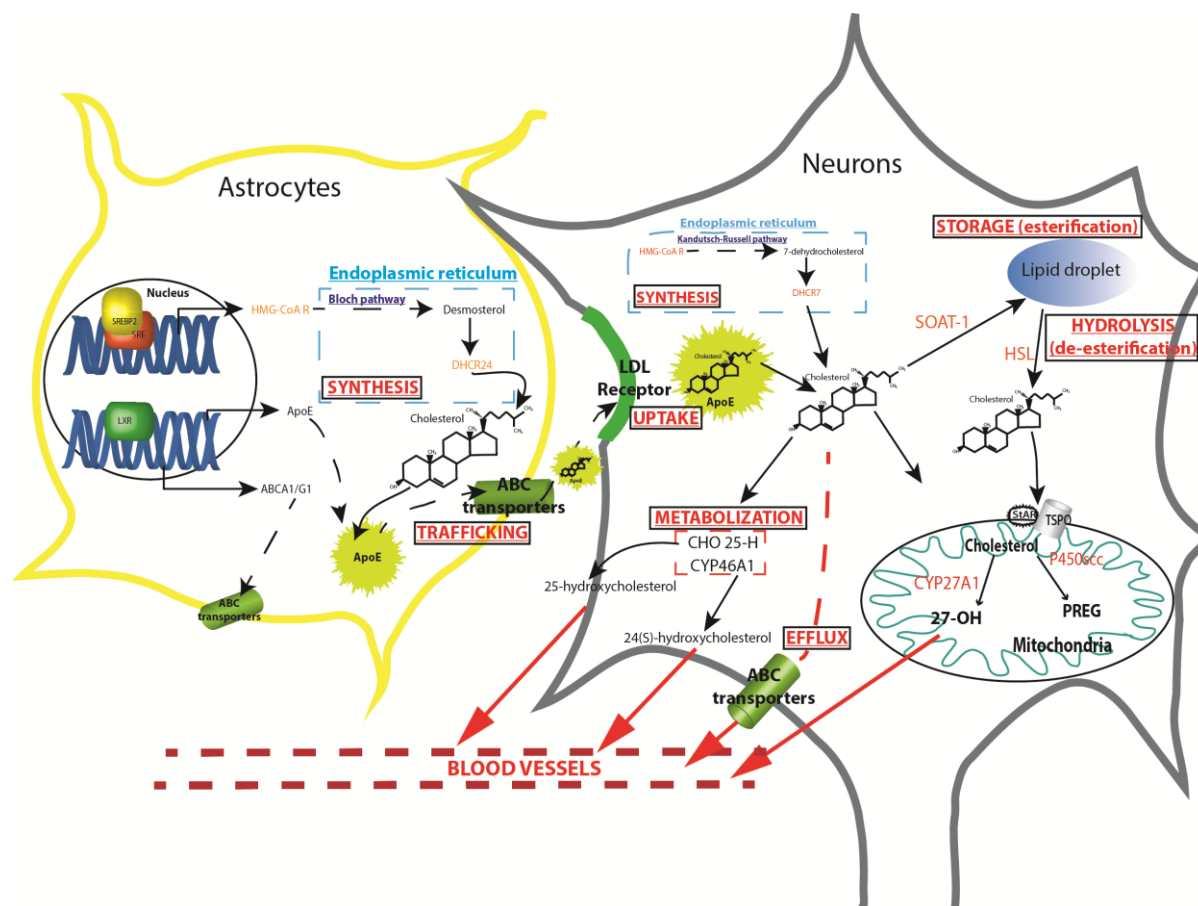
Figure 9. Brain cholesterol homeostasis. Sterol regulatory element-binding protein 2 (SREBP-2); Sterol responsive-element (SRE); Liver X receptor (LXR); 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA R); 24-dehydrocholesterol reductase (DHCR24); 7-dehydrocholesterol reductase (DHCR7); Apolipoprotein E (ApoE); ATP-binding cassette (ABC) A1/G1; Sterol-o-acyl transferase (SOAT-1); Hormone sensitive lipase (HSL); Cholesterol 25-hydroxylase (CHO 25-H); Cholesterol 24-hydroxylase (CYP46A1); Cholesterol 27-hydroxylase (CYP27A1); Steroid acute regulatory protein (StAR); 18 kDa translocator protein (TSPO); Cytochrome P450 side chain cleavage (P450scc). This diagram summarizes the homeostasis of cholesterol. In the CNS, the homeostasis of cholesterol is composed by a synergic actions of synthesis (by astrocytes and neurons), storage, metabolism and trafficking (and/or efflux). In particular, neurons and glial cells may handle excess cholesterol by esterification (SOAT-1) and subsequent intracellular storage, or by direct excretion via ABC transporters and/or by conversion to enzymatic oxysterols such as 24(S)-OH, 25-OH and 27-OH. Finally, cholesterol may be metabolized by P450scc into PREG, the precursor of neuroactive steroids.

Mitochondria

As previously mentioned, steroidogenesis requires as first step the translocation of cholesterol from the cytoplasm to the inner mitochondrial membrane (IMM). Then cholesterol is available to the first enzyme of steroidogenesis which is in located in the mitochondrial compartment. These organelles are not only the subcellular compartment where steroidogenesis occurs. Indeed, mitochondria are involved in the response to pathological conditions that cause stress to the energy metabolism. Although their most well-known function is cellular energy production and conservation, mitochondria are central to cell cycle regulation, are involved in programmed cell death, calcium signaling, and redox homeostasis and signaling.

Mitochondria are symbiotic organelles that contain their own genetic material, the mDNA, which encodes essential subunits of the respiratory chain complex I, III, IV, and V. At complex I, electrons derived from energetic substrates (glucose and lipids) enter the respiratory chain and travel to complex IV, where they are combined with oxygen to produce energy in the form of ATP required for life (Wallace et al., 2010). ATP generated inside mitochondria is exported from the INN to the cytoplasm by adenine nucleotide translocators (ANTs), where it fuels the energy-dependent cellular reactions. During electron flow through the respiratory chain, reactive oxygen species (ROS) are generated, leading to oxidative stress when antioxidant defenses are insufficient (Lambert and Brand, 2009).

Mitochondrial bioenergetics system

The mitochondrial electron-transport system consists of several multi-polypeptide protein complexes (I-V) embedded in the IMM that receive electrons from reducing coenzyme such as NADH and $FADH_2$ (Figure 10). These electrons are transferred through a series of electron carriers in the respiratory chain, where O_2 serving as the final electron acceptor and is reduced ultimately into H_2O. Each of the electron carriers represents a redox couple (i.e. species capable of existing in a reduced or oxidized state) with a characteristic reduction potential. The electron carriers in the respiratory chain are ordered so that the reduction potentials progressively increase (i.e. become more positive) from one redox couple to another. In three of these complexes (I, III and IV), the difference in reduction potential (i.e. release of energy), across successive redox couples, is sufficient to drive the translocation of protons from the

matrix to the inner membrane space. The energy release creates a proton gradient across the inner membrane that is derived from both the concentration and the electrical potential difference across the membrane. The essence of the chemiosmotic theory is that the electrochemical energy created by the generation of the proton motive force is sufficient to drive the synthesis of ATP as well as to flow back, through the ATP synthase complex, the protons into the matrix (Fisher-Wellman and Neufer, 2012). The transport of electrons through the respiratory chain is an inherent property of the system – it occurs automatically. For the most part, electron flow and proton pumping are tightly coupled; that is one does not occur without the other. To activate respiration, fuel (i.e. NADH, $FADH_2$) is added which automatically initiates electron flow, proton pumping, and O_2 consumption. However, to regulate this process on the outer surface of the inner membrane, a "back pressure" is created which begins to oppose the pumping of protons, thereby gradually slowing electron flow and O_2 consumption. Mitochondria should never reach a state of "static head" equilibrium, where the force driving the pumping of protons out the matrix is completely counter-balanced by the high membrane potential. This basal rate of proton conductance ensures that the membrane potential is almost less than maximum, "allowing" electron flow, proton pumping, and O_2 consumption, to operate at an "idling" rate (known as state 2 respiration). When another flux of proton is created to flow back into the matrix (e.g. via ATP synthase catalyzing re-phosphorylation of ADP), the back pressure of the membrane potential is further reduced accordingly with the proton pumping and oxygen consumption increase (known as state 3 respiration). Once all of the ATP is synthesized, the system slows back to the idling rate (known as state 4 respiration). The crucial point is that the mitochondrial respiratory system is a "primer engine", which automatically adjusts to each change in the rate of proton re-entry into the matrix, a corresponding change in electron flow and O_2 consumption. The respiratory system operates is governed by energy demand, and not by energy supply. Proton conductance (via leak and ATP synthase) dictates the rate of electron flow and therefore the demand for reducing equivalents (e.g. NADH and $FADH_2$) that, in turn, regulates the rate of substrate uptake and flux through catabolic pathways (Fisher-Wellman and Neufer, 2012).

The product of the reduction potential and the reducing capacity (i.e. the concentration of the reduced species) of a redox couple is the definition of the redox state. Several of the redox couples within the electron-transport chain transfer single rather than two electrons and are

therefore susceptible to leaking electrons directly to surrounding O_2 to form the free-radical superoxide (O_2^-). Under state 3 conditions, the redox state of the system is below the threshold at which electrons will leak to O_2. However, at or near state 4, the respiratory system is in its most reduced state such that the rate at which O_2^- is produced is extremely sensitive to the redox state of the system, increasing exponentially with even small increases in membrane potential (Korshunov et al., 1997, Liu, 1997, 1999). Fortunately, O_2^- is rapidly converted by manganese superoxide dismutase (MnSOD or SOD-2) to the two-electron non-radical hydrogen peroxide (H_2O_2). H_2O_2 in turn can be further reduced to H_2O in mitochondrial matrix by glutathione (GSH) or the thioredoxin/peroxiredoxin systems, or can freely diffuse out of the mitochondrial were it again is buffered by GSH. Thus in a resting cell, the mitochondrial respiratory system functions as redox pressure-gauge that senses and reflects cellular metabolic balance. When in positive balance, electron leak serves as release valve, accelerating mitochondrial O_2^- production and H_2O_2 emission (Fisher-Wellman and Neufer, 2012).

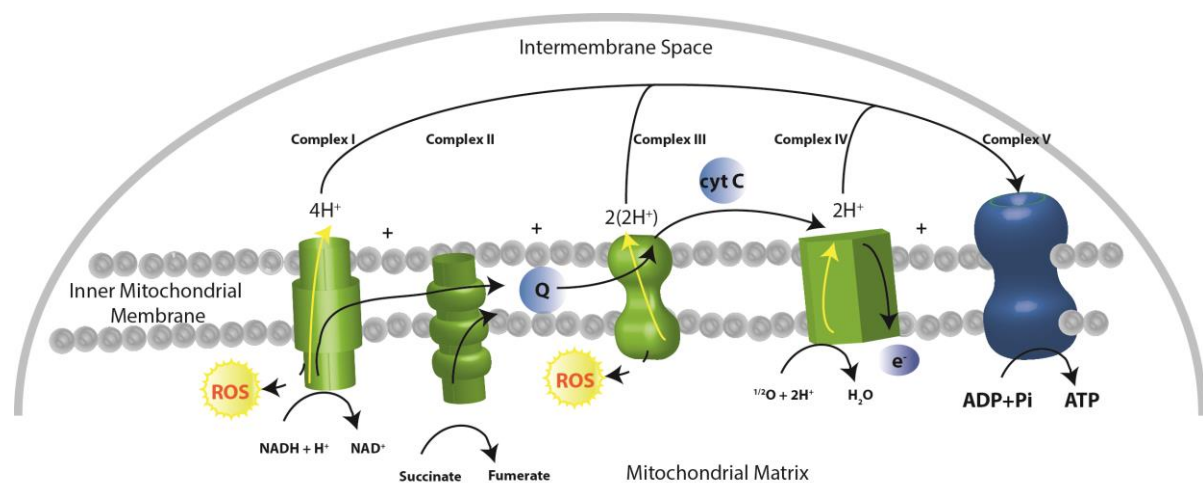**Figure 10. Mitochondrial electron-transport chain.** The transport chain in the mitochondrion is the site of oxidative phosphorylation. The NADH and succinate generated in the citric acid are oxidized (Complex I and Complex II), providing energy to power ATP synthase (Complex V). The reactive oxygen species (ROS) are byproduct of this process while O_2 is the final acceptor of the electron-transport chain

Reactive oxygen species in neurodegeneration disease

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

Mitochondrial metabolism is also involved in the intracellular ROS production. Indeed, the unpaired electrons leave the ETS, react with molecular oxygen and produce superoxide anions. This class of molecules further interacts with lipids, DNA and proteins and play crucial role in numerous signaling processes, which is associated with the disease phenomenon of both aging and neurodegenerative disorders (NDDs) (Uttara et al., 2009). Moreover, ROS can also react with nitric oxide (NO) to produce reactive nitrogen species (RNS) (Patel et al., 1999). There are several NDDs identified so far, that have been reported to be associated with stress and mitochondrial dysfunction, which include Alzheimer's disease, Parkinson's disease, Huntington's disease and Amyotrophic lateral sclerosis (Seo et al., 2010).

Overproduction of ROS, as byproducts generated from electron flow through the respiratory chain, usually occurs during normal respiration where the $1 - 6$ % of the oxygen reduced by mitochondria is converted to superoxide anion at the level of complex I or at the level of ubiquinone (Chance et al., 1979, Boss et al., 1997, Bechmann et al., 2002). Normally cells convert O_2^- to H_2O_2 utilizing both SOD-2, which is encoded by a nuclear gene but localized to the mitochondria compartment, and copper-zinc superoxide dismutase (SOD-1) founded in the cytosol. Superoxide anions do not readily cross membranes (Fridovich, 1986), but may be transported by anion channels (Kontos et al., 1985). Superoxide anions rapidly react with NO, forming peroxynitrite ($ONOO^-$), which is a mediator of neurodegeneration (Estevez et al., 1995, Schulz et al., 1995, Szabo, 1996), that may damage and kill cells by induction of lipid peroxidation and protein tyrosine nitration (Beckman, 1994, Beckman et al., 1994). Hydrogen peroxide easily penetrates lipid bilayers, acts as an oxidizing agent, and is relatively stable,

although it is not a free radical. Moreover, this molecule helps modulate signaling system in the cell, such as kinase and phosphatase (Whisler et al., 1995, Denu and Tanner, 1998) and transcription of genes (Mattson and Camandola, 2001). Hydrogen peroxide is not toxic except in high concentration of hydroxyl radicals (O_2^- + H_2O_2 -> OH + OH^- + O_2), particularly in the presence of ferrous ions, which are present in the brain parenchyma (e.g. after trauma and intracerebral hemorrhage). Hydroxyl radical are extremely reactive, and rapidly attack unsaturated fatty acids in membranes causing lipid peroxidation and the production of 4-hydroxynonenal that conjugates to membrane proteins, impairing their function (Keller et al., 1997a, Keller et al., 1997b). During homeostasis, the production of ROS is balanced by anti-oxidant system such as SOD, catalase, glutathione peroxidase and glutathione reductase, maintaining the levels of $O_2^{\bullet -}$ and H_2O_2 in vivo at about 10^{-11} and 10^{-9} M respectively (Forman and Kennedy, 1974, Forman and Wilson, 1982).

As previously mentioned, mitochondrial ROS production is intimately linked to membrane potential. Indeed, at high membrane potentials, protons cannot be pumped out of the matrix (against the electrochemical proton gradient) by the electron transport chain. As a consequence, the electron transport slows, thus resulting in the production of intermediates able to reduce O_2. These processes increase ROS production that may be reduced, modestly, by the mitochondrial inner membrane through the uncoupling proteins (UCPs) (Skulachev, 1996, Kim-Han et al., 2001, Votyakova and Reynolds, 2001).

The UCPs are encoded by nuclear DNA and are located in the IMM. Their primary function is thought to be to translocate protons from the intermembrane space to the matrix of the mitochondria (Bouillaud et al., 1985, Fleury et al., 1997, Vidal-Puig et al., 1997, Sanchis et al., 1998, Mao et al., 1999, Azarashvili et al., 2003). In the individual mitochondrion, these proteins, through this process, may reduce the driving force of ATP synthase from catalyzing ATP synthesis, dissipate energy in the form of heat, diminish the production of superoxide anion, and decrease the likelihood of calcium entry to the mitochondrial matrix (Negre-Salvayre et al., 1997, Arsenijevic et al., 2000, Lowell and Spiegelman, 2000). Also, UCPs appear to be important to several metabolic processes (Klingenberg and Echtay, 2001). For instance, UCP2 acts as a protonophore and is activated by superoxide anions from within the matrix of the mitochondria (Echtay et al., 2002). Several studies have reported roles for UCPs in modulating ROS production (Negre-Salvayre et al., 1997, Arsenijevic et al., 2000, Sullivan et

al., 2003, Sullivan et al., 2004a, Sullivan et al., 2004b). For instance, leptin-deficient mice have decreased levels of UCP and increased ROS production in macrophages (Lee et al., 1999). Overexpression of UCP2 decreases cell death following H_2O_2 exposure and ROS production (Li et al., 2001). This protein is express in various part of the brain and may play a role in neuroendocrine, behavioral, autonomic functions and metabolic processes (Horvath et al., 1999, Diano et al., 2000, Richard et al., 2001).

Mitochondrial biogenesis

As previously mentioned mitochondria play a vital cellular role in the maintaining of energy homeostasis. Moreover, these organelles are also involved in the response to oxidative stress produced in the pathological conditions as well as in cell cycle regulation, programmed cell death, fatty acid and cholesterol homeostasis, and steroid biosynthesis. All these functions needed a finely regulation of the mitochondrial functions, numbers and distribution. Mitophagy, mitochondrial fission, fusion, and biogenesis may occur to optimize the mitochondrial physiology (Zhu et al., 2013, Palikaras and Tavernarakis, 2014). Mitochondrial biogenesis is a process metabolically expensive because it may involve the synthesis of hundreds or, in some cases, thousands of new proteins. As a result, mitochondrial biogenesis is strictly controlled by intra- or extracellular signals communicating energy imbalance due to increasing energy demand, decreased energy production or both. Mitochondrial biogenesis can be induced by exercise, fasting, cold exposure (thermogenesis), oxidative stress, and inflammatory cell stress. Depending on the stimulus, the program is executed through a variety of pathways that converge on few coactivators and nuclear transcription factors such as the peroxisome proliferator-activated receptor gamma-1 (PGC-1) coactivator family and nuclear respiratory factors (NRF). In particular, PGC-1α has been identified as an important coordinator of the biogenesis response and has been found to orchestrate a wide variety of anti-inflammatory and metabolic nuclear genes (Wu et al., 1999, Valle et al., 2005, Cherry et al., 2014). These transcription factors and coactivators coordinate the complex bio-genomic programs of biogenesis by participating in feedback loops for the precise regulation of mitochondrial quality control and modulating specific gene expressions at various regulatory levels within the process (Cherry and Piantadosi, 2015). For instance, NRFs proteins upregulate the transcription of many nuclear-encoded mitochondrial proteins, which are transported across the mitochondrial membranes. These imported proteins serve as the

building blocks for mitochondrial proliferation, while the same central coactivators and transcription factors upregulate expression of mt-DNA-binding proteins such as the mitochondrial transcription factor A (TFAM). In other words, PGC-1α increases the expression and acts as a coactivator for TFAM, which is mainly responsible of the transcription and replication of mitochondrial genes from the mitochondrial genome (Wu et al., 1999).

It is interesting to note that the role of PGC-1α, as transcriptional coactivator, is not restricted to the mitochondrial biogenesis. Indeed, PGC-1α plays a pivotal role in a wide range of biochemical events, including gluconeogenesis and glucose metabolism (Lin et al., 2002, Puigserver and Spiegelman, 2003). In addition, PGC-1α is a positive regulator of diverse mitochondrial, peroxisomal and ROS-detoxifying gene networks. The elevated expression of these genes in cells that ectopically express PGC-1α has been mostly attributed to increased mitochondrial biogenesis. Indeed, it is expected that if an enzyme is located in mitochondria and there is a proliferation of mitochondria, the cellular content of this enzyme may increase. However, there is growing evidence that PGC-1α also modulates the intrinsic composition of mitochondria. In other words, the new mitochondria that are generated in the presence of PGC-1α have different properties compared with the original organelles. These changes in the intrinsic properties of mitochondria will have a central impact on cellular gene expression profiles and oxidative metabolism (Austin and St-Pierre, 2012).

Several studies have demonstrated that PGC-1α has been implicated in many pathological conditions, such as diabetes and heart disease (Handschin and Spiegelman, 2006). For example, PGC-1α expression is reduced by several neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis and Duchenne muscular dystrophy (Qin et al., 2009, Gong et al., 2010, Sheng et al., 2012). On the contrary, several studies support the protective role for PGC-1α in Parkinson's disease that is characterized by the loss of dopaminergic neurons with the consequent decreased PGC-1α target genes (Zheng et al., 2010). Indeed, PGC-1α protects against neuronal loss in cell culture models of Parkinson's disease (Wareski et al., 2009, Zheng et al., 2010).

Mitochondrial dysfunction in cholesterol efflux

Trafficking of cholesterol form the outer to the cholesterol-poor IMM is established as the rate-limiting step in provision of cholesterol substrate to the cytochrome P450scc which, as

previously mentioned, is responsible for production of PREG, the common precursor of steroid hormones (Manna et al., 2009). This process is also the rate-limiting step governing the activity of CYP27A1 (Bjorkhem et al., 1999), which converts cholesterol into 27-hydroxycholesterol (Allen et al., 2013). In the current model, cholesterol is transferred to the IMM by a protein complex, composed by TSPO, voltage-dependent anion channel (VDAC) and StAR, that forms contact sites between the outer and inner mitochondrial membranes.

Mitochondrial cholesterol levels influence bioenergetics. However, the underlying mechanisms and the overall consequence for energy homeostasis vary among model system and are not yet fully understood. Several studies have found decrease in ATP synthesis, hydrolysis or respiration in mitochondrial enriched with cholesterol (Martin et al., 2016). Other studies show that mitochondrial cholesterol accumulation was associated with increased ROS production (Llacuna et al., 2011).

Studies in which pharmacological agents were used to disrupt mitochondrial function have suggested that energized, polarized and actively respiring mitochondria are required to sustain mitochondrial cholesterol transport and steroid hormone biosynthesis (Hales et al., 2005, Allen et al., 2006). Moreover, perturbation in mitochondrial function may alter the expression of CYP27A1. Indeed, high levels of ROS are linked to reduce TSPO expression and cell death, which may allow overt and unregulated production of 27-hydroxycholesterol, causing further mitochondrial damage and apoptosis (Armstrong, 2007). It is evident that steroidogenesis is highly sensitive to perturbed mitochondrial function.

Mitochondrial cholesterol levels are increased in the brain of a murine model of Alzheimer's disease (Fernandez et al., 2009, Barbero-Camps et al., 2013, Barbero-Camps et al., 2014). In many cases, the increased of the mitochondrial cholesterol import is due to increase in cholesterol availability, for example due to endosomal cholesterol accumulation or a decreased activity of the enzyme into the mitochondrion.

Even if mitochondrial cholesterol accumulation is largely a secondary effect, it still has consequence for mitochondrial function. These alterations increase the sensitivity to other insults, such as oxidative stress (Martin et al., 2016).

Diabetes encephalopathy

Diabetes is a chronic metabolic disorder characterized by high plasma glucose levels (≥ 300 mg/dl). Around 220 million people worldwide have diabetic disease, and this number is estimated to double by 2030 due to urbanization, obesity and aging (Wild et al., 2004). In the United States, approximately 25,8 million children and adults have diabetes (ADA, 2011). Because of its chronic nature, the development of long-term complications, and the progressive increase in young people, diabetes represents one of the most expensive disease in the world (IDF - international diabetes federations). Different types of diabetes have been characterized, but the most common forms are type 1 diabetes, characterized by a partial or total loss of insulin secretion, and type 2 diabetes, which is characterized by reduced insulin sensitivity and consequently insulin deficiency. Type 1 diabetes represents approximately 5 – 10% of all diagnosed cases of diabetes and affect people of any age, but usually develops in children or young adults. This condition is characterized by a massive destruction of pancreatic β-cells, leading to insulin deficiency (Celotti, 2006, NDIC, 2011). Type 2 diabetes is characterized by insulin resistance due to obesity, sedentary life style, and genetic predisposition (Lazar, 2005). Age is also a risk factor for insulin resistance, with older adults developing type 2 diabetes at higher rate than middle aged or younger populations (DeFronzo, 1981). In the industrialized and developing countries, the incidence of this form of diabetes is increased in the last decades until represent the 90% of diabetic cases in the world (Celotti, 2006). Both forms are associated with hyper- and hypoglycemia, glycosuria, polyuria, polydipsia, polyphagia, loss of weight, metabolic acidosis, and alteration of carbohydrate, cholesterol and lipid metabolism. Moreover, chronic hyperglycemia can lead to microvascular and macrovascular complications, which may affect the kidney, eyes, and nervous system.

The most frequent symptomatic complication is the damage at the levels of peripheral nervous system (i.e. diabetic neuropathy) that is characterized by a spectrum of functional and structural changes in peripheral nerves including axonal degeneration and decreased nerve conduction velocity (Sugimoto et al., 2000, Vinik et al., 2000). Neurophysiological and structural changes also have been reported in the central nervous system (CNS) and are called diabetic encephalopathy (DE) (McCall, 2002). These alterations are associated with cognitive deficits and increase risk of dementia, stroke, cerebrovascular and Alzheimer disease and

psychiatric disorders, such as depression, and eating disorders (Gispen and Biessels, 2000, Biessels et al., 2002, Jacobson et al., 2002, Biessels et al., 2008, Kodl and Seaquist, 2008). DE represent the long-term neurological complication of diabetes (Biessels and Gispen, 2005) its prevalence is 40% in long standing and poorly controlled diabetes (Dejgaard et al., 1991). Hyperglycemia-induced cognitive decline has shown to be associated with severe cortical atrophy and vascular lesions in patients (Manschot et al., 2007).

Glucose Excitotoxicity

The brain uses glucose as preferential energy source since it is not metabolically adjusted for the metabolism of free fatty acids (Pardridge et al., 1990). Brain glucose uptake appears to be independent of insulin action (Peters et al., 2004). The predominant transporters involved in subsequent glucose uptake from the extracellular fluid in neurons and astrocytes are GLUT3 and GLUT1, both insulin-independent (Wilson, 2003, Simpson et al., 2007). GLUT1 and GLUT3 is highly expressed in the hippocampus, the cerebellum and the olfactory bulb. However, the insulin-sensitive glucose transporter, GLUT4, is also expressed in several regions of the brain. Finally, mRNA coding for glucokinase and the insulin receptor (IR), are also expressed in some of these brain regions (Peters et al., 2004). The normal fate of intracellular glucose is phosphorylation of the number-six-position carbon by hexokinase, and entry into glycolysis. However, when insulin independent uptake is driven by abnormal high levels of glucose in the interstitial fluid, the glucose is diverted to metabolic pathways that can result in neurotoxicity (Tomlinson and Gardiner, 2008). The hyperglycemic status, in diabetes, causes up to fourfold increases in neuronal glucose levels. If this is persistent, or if such episodes are regular events, the intracellular glucose metabolism leads to neuronal damage; this phenomenon is often referred to as glucose neurotoxicity (Tomlinson and Gardiner, 2008). Several hypotheses of glucose neurotoxicity have been proposed such as glucose-driven oxidative stress, protein glycation, and alteration of the polyol (sorbitol) pathway and the intracellular signals (Tomlinson and Gardiner, 2008). On the other hand, the chain of events leading from these molecular changes to the functional outcomes is less clear, because most of the putative dysfunctions cannot be demonstrated in cell culture and it is difficult to be certain of the molecular antecedents of function in diabetic models in vivo (Tomlinson and Gardiner, 2008).

Diabetes induces oxidative stress

As previously mentioned one of the mechanisms of glucose neurotoxicity is the oxidative stress. The production of reactive oxygen species (ROS) is promoted by glucose through a combination of free radical generation and impaired free-radical scavenging. Hydrogen peroxide is produced by the action of superoxide dismutase on superoxide (O_2^-), which is itself generated by increased oxidative metabolism of glucose in the mitochondria (Nishikawa et al., 2000b). Moreover, the sorbitol pathway compromises the glutathione cycle by consuming the proton donor NADPH. This reduces the capacity of glutathione peroxidase to metabolize hydrogen peroxide to water (Obrosova et al., 2002). Briefly, both systemic glucose and lipid metabolism converge in mitochondria generating the majority of cellular energy (ATP) by coupling the tricarboxylic acid cycle (TCA) cycle with oxidative phosphorylation (OXPHOS). Acetyl-CoA generated from glycolysis (glucose) and fatty acid β-oxidation (lipid) enters the TCA cycle in the mitochondrial matrix, in which the substrates are oxidized with the formation of CO_2, NADH and $FADH_2$. The electron from NADH and $FADH_2$ are taken up by the respiratory chain complexes which generates an electrochemical gradient and drives the electron to ATP generation in the complex V. Mitochondrial metabolism is responsible for the major energy supply to vital cell functions including the maintenance of transmembrane ion gradients, protein synthesis, and vesicular transport (Wallace, 1999). Normally, only 0.1% of total oxygen consumption leaks from the respiratory chain to generate ROS. However, during the diabetic disease the hyperglycemia increase the electron flux through the mitochondrial electron transport chain. Consequently, there is an increase of the ATP/ADP ratio and hyperpolarization of the mitochondrial membrane potential. This high electrochemical potential difference generated by the proton gradient leads to partial inhibition of the electron transport in complex III, resulting in an accumulation of electrons to coenzyme Q. In turn, this drives partial reduction of O_2 to generate the free radical anion superoxide (Nishikawa et al., 2000a, Brownlee, 2001).

As previously mentioned, hyperglycemic status, reduces antioxidant levels and concomitantly increases the production of free radicals. These effects contribute to tissue damage in diabetes mellitus, leading to alterations in the redox potential of the cell with subsequent activation of redox-sensitive genes (Muriach et al., 2014). As a result of its high oxygen consumption rate, abundant lipid content, and the relative paucity of antioxidant enzymes as

compared to other tissues, the brain is especially vulnerable to oxidative stress damage. Nevertheless, under normal condition, a balance exists between the production of ROS and the antioxidant mechanisms (Bala et al., 2006), when this balancing mechanism fails, such as in diabetes, oxidative insults and therefore ROS may contribute to neurodegenerative processes (Jackson et al., 1994, Dugan et al., 1995, Yuan and Yankner, 2000). In agreement, Cardoso et al. (2013), have shown that hippocampal mitochondria of STZ rats presented higher levels of lipid peroxidation, a marker of oxidative stress. Together with this data, the activity of some proteins involved in the antioxidant defense was altered by diabetic disease. Indeed, the activity of superoxide dismutase 2 (SOD-2) (i.e. the mitochondrial isoform of an antioxidant enzyme) was decreased after three months of diabetes (i.e. long-term). However, the activity of glutathione disulfide, which balances the production of H_2O_2, is increased while the glutathione-to-glutathione disulfide ratio (i.e. an index of oxidative stress) is decreased by long-term diabetes. This dichotomy between antioxidant molecules could be an attempt to overcome the decreased SOD-2 activity in order to maintain unchanged the H_2O_2 levels (Cardoso et al., 2013). It also showed impaired oxidative phosphorylation system characterized by a decreased mitochondrial energization potential and ATP levels and higher repolarization lag phase (Cardoso et al., 2013). In accordance with these data, Ortiz et al. (2013) has reported that diabetes increases lipid peroxidation and mitochondrial superoxide anions associated with an altered mitochondrial respiratory function in the brain cortex of STZ rats.

Effects of diabetes on cholesterol homeostasis

As previously mentioned, the brain is a very cholesterol-rich organ, which is important in several processes, such as the synaptogenesis, the maintenance of membrane fluidity, vesicle formation and the biosynthesis of steroids. Diabetes can alter the cholesterol homeostasis in the brain. In vitro experiments co-culturing astrocytes and neurons suggest that cholesterol is synthesized in astrocytes, packaged into ApoE particles, secreted to be taken up by neurons and incorporated into membranes to augment synaptogenesis and vesicle formation (Mauch et al., 2001). The majority of cholesterol present in CNS (70%) is in myelin sheaths and turns over very slowly (Barres, 2008). Most of the remainder is in the membranes of astrocytes and neurons (Bjorkhem and Meaney, 2004). This pool turns over more quickly (Okabe et al., 1999) and is therefore likely to be most reflective of short-term changes in cholesterol synthesis.

Impairment of myelin formation has been observed in the brain of mouse models of long-term type 1 diabetes (Francis et al., 2008). Cholesterol metabolism in the brain plays an important role in myelin production (Dietschy and Turley, 2004) and it has been implicated in regulation of many processes (Mitter et al., 2003). Several reports have shown that diabetes increases the cholesterol levels in the liver, kidney and heart of type 2 diabetic rats (Stanely Mainzen Prince and Kannan, 2006, Geethan and Prince, 2008). Alterations of cholesterol levels in tissues may be a factor contributing to the diabetic complications. Decreased cholesterol biosynthesis molecules, such as HMG-CoA R, due to a down-regulation of SREBP-2 (i.e. the main regulator of the cholesterol biosynthesis) occur in diabetic mice (Suzuki et al., 2010). These mice also have decreased levels of SCAP (i.e. a sterol-sensing molecule), which contributes to an altered processing of immature SREBP-2 to its mature form, leading to a further defect in cholesterol synthesis (Suzuki et al., 2010, Suzuki et al., 2013). This study raises an important question about the therapy with statins (HMG-CoA R inhibitors) that might alter the brain function in normal individuals or patients with diabetes or dementia. Some studies have reported relatively acute cognitive decline and memory loss in individuals on statins, which improve after discontinuation of the drug (Xiong et al., 2005, Tuccori et al., 2008, Evans and Golomb, 2009). On the other hand, an important way to maintain cellular cholesterol homeostasis is the trafficking via cholesterol transporters such as ABCA1 and ABCG1, which are important rate-controlling protein (Calkin et al., 2005, Tang et al., 2010). Wang et al. (2012) showed that the expression of ABCA1 increased in hippocampus and cerebral cortex of STZ-induced diabetic rats. Indeed, a report showed that insulin could enhance the degradation of ABCA1 and downregulate its activity by promoting the phosphorylation of the protein (Nonomura et al., 2011).

Diabetes alters neuroactive steroids

Experimental diabetic neuropathy and experimental diabetic encephalopathy show similar features to human complications (Biessels et al., 1999, Bianchi et al., 2004, Biessels and Gispen, 2005, Mastrocola et al., 2005, Beauquis et al., 2006, Stranahan et al., 2008, Zhang et al., 2008, Alvarez et al., 2009). Recent findings from experimental models of diabetic neuropathy and diabetic encephalopathy indicate that neuroactive steroids are protective agents (Aragno et al., 2002, Yorek et al., 2002, Saravia et al., 2004, Saravia et al., 2006, Veiga et al., 2006, Leonelli et al., 2007, Beauquis et al., 2008, Roglio et al., 2008, De Nicola et al.,

2009). For instance, treatment with PROG, or its 5α-reduced metabolite, DHP, counteracts the increase in the number of fibers with myelin infoldings observed in the sciatic nerve of STZ-treated rat (Veiga et al., 2006). Moreover PROG, T and their derivatives (e.g. DHP, THP, DHT and 3α-diol) or dehydroepiandrosterone (DHEA) influence a variety of biochemical and functional parameters, including nerve conduction velocity, thermal threshold, skin innervation density, Na+,K+-ATPase activity and expression of myelin proteins, which are affected in STZ-treated rats (Yorek et al., 2002, Leonelli et al., 2007, Roglio et al., 2008). In the CNS, DHEA protects the hippocampus from diabetic damage reducing nuclear factor-kappa B nuclear translocation (Aragno et al., 2002).

On the other hand, another important aspect of neuroactive steroids, is that their levels are affected in the plasma of the long-term model of STZ-rat due to dysfunction in the reproductive axis (Babichev et al., 1998, Durant et al., 1998, Tanaka et al., 2001, van Dam et al., 2003, Salonia et al., 2006, Leonelli et al., 2007, Roglio et al., 2008). Moreover, the disease also affects the local levels of neuroactive steroids in the PNS (Leonelli et al., 2007, Caruso et al., 2008b, Roglio et al., 2008). Recent findings have reported, in a long term model of diabetes (i.e. three month of diabetes), that alterations of neuroactive steroid levels in three brain areas of the CNS occurs. In particular, cerebral cortex (CTX), cerebellum (CB) and spinal cord (SC) were assessed. Data reported decreased levels of several neuroactive steroids such as PREG, PROG, DHP, THP, ISOPREG, T, DHT, 3α-diol in the CTX of STZ rats. Moreover, also the neuroactive steroids in the CB of diabetic rats were altered in particular, the levels of PREG, DHP, T and DHT were affected. Finally, decreased levels of PREG, PROG, DHP, THP, T, DHT and 3α-diol occur in the spinal cord after three months of diabetes (Pesaresi et al., 2010, Mitro et al., 2012)

Effects of diabetic encephalopathy

In human with either type 1 or type 2 diabetes, imaging studies have demonstrated smaller hippocampi, as well as changes in the functional connectivity between regions of the brain (Bolo et al., 2011, Musen et al., 2012, Antenor-Dorsey et al., 2013, Lyoo et al., 2013). Moreover, type 1 diabetes has a specific effect on a subset of cognitive domains in adults, including intelligence, attention, psychomotor speed, cognitive flexibility and visual perception (Brands et al., 2005). In type 1 diabetes, cognitive dysfunction emerges early in the

disease course (within 2 years of diagnosis). Age is also an important variable, and children's brains might be more susceptible to the effects of diabetes than adult's brains. In particular, individuals who develop type 1 diabetes early in life (young than 7 years) have a higher risk of developing more severe cognitive deficits than are those who develop diabetes at an older age (Ryan, 2006).

The hippocampus is a crucial part of the limbic system, which plays a pivotal role in memory formation, emotional, adaptive, and reproductive behaviors (Squire, 1992, Witter, 2004). This brain region is also particularly important to connect emotions and senses, such as smell and sound, to memories (Turgut and Turgut, 2011). The structural complexity of hippocampus has made it vulnerable to the many pathological conditions and metabolic disorders including diabetes mellitus (Biessels et al., 1996, Alvarez et al., 2009, Pamidi and Satheesha Nayak, 2012, Foghi and Ahmadpour, 2013). Indeed, Li et al. have reported hippocampal neuronal death in spontaneous rat model of type 1 diabetes mellitus, accompanied by some functional cognitive alteration after a long period of diabetes (8 months) (Li et al., 2002c). Moreover, rats and mice, raised diabetic with a single i.p. injection of STZ, shows a decrease hippocampal cell proliferation (Jackson-Guilford et al., 2000, Saravia et al., 2004, Beauquis et al., 2006, Kang et al., 2006, Stranahan et al., 2008, Zhang et al., 2008, Balu et al., 2009, Revsin et al., 2009, Wang et al., 2009a, Wang et al., 2009b, Piazza et al., 2011).

In the recent years, increasing evidence of cognitive dysfunction as well as memory impairment caused by diabetes, has been obtained in animal models (Biessels et al., 1996, Li et al., 2002b). In STZ mice and rats, model of type 1 diabetes, neurobehavioral deficits have been detected whit the Morris water maze test, which is a spatial memory assay. These animals displayed also impaired hippocampal long-term potentiation (Biessels et al., 1996). Numerous studies have shown that experimental diabetes has negative impacts and induce apoptosis in hippocampal neurons via multiple mechanisms. However, the molecular basis of these complications is poorly understood.

Aim

Diabetic disease is a chronic metabolic syndrome characterized by hyperglycemia, alterations in carbohydrate, lipid and protein metabolism (Suzuki et al., 2010, Wang et al., 2012). Diabetes may induce neurophysiological and structural changes at the level of central nervous system (CNS) areas (i.e., diabetic encephalopathy) (McCall, 2002). These complications are associated with acute alterations in mental status due to poor metabolic control, decline in cognitive function, increase risk of dementia and psychiatric disorders such as depression and eating disorders (Gispen and Biessels, 2000, Jacobson et al., 2002, Cukierman et al., 2005, Biessels et al., 2008, Kodl and Seaquist, 2008).

Data in literature have demonstrated that diabetic encephalopathy alters neuroactive steroids levels in cerebral cortex, cerebellum and spinal cord of a rat model of type 1 diabetes after three months (i.e. long-term diabetes) (Pesaresi et al., 2010).

Evidence in literature suggests that diabetic encephalopathy may already occur after short-term period of diabetes (Chabot et al., 1997, Cukierman et al., 2005, Kodl and Seaquist, 2008, Revsin et al., 2009, Suzuki et al., 2010). Therefore, on the basis of our previous data obtained in long-term diabetes on neuroactive steroid levels (Caruso et al., 2008a, Pesaresi et al., 2010), our aim will be to assess whether, in the hippocampus, cerebral cortex and in plasma of STZ rats, the levels of neuroactive steroids are already altered after one month of diabetes. On the basis of the results obtained we measured in the hippocampus and cerebral cortex the gene expression of molecules involved in synthesis and metabolism of neuroactive steroids as well as of cholesterol (i.e. the substrate in the synthesis of neuroactive steroids). In this contest it is important to remember that the brain is the most cholesterol-rich organ, where de novo synthesis accounts for the major part of cholesterol here present and the blood brain barrier prevents cholesterol uptake from the circulation (Bjorkhem and Meaney, 2004, Dietschy and Turley, 2004). Moreover, because the limiting step of steroidogenesis occurs in the mitochondria, and their function is affected by oxidative stress occurring in diabetic encephalopathy (Singh et al., 2004, Ceretta et al., 2012, Ortiz et al., 2013) we assessed the effects of short-term diabetes on the mitochondria functionality in the hippocampus and cerebral cortex.

Methods

Animals

Male Sprague-Dawley rats (175-200 g at arrival, Charles River Laboratories, Lecco, Italy) where used. Animals were housed in the animal care facility of the Dipartimento di Scienze Farmacologiche e Biomolecolari (Università degli studi di Milano). All animals were kept in standard rat cages with food and tap water available ad libitum and under controlled temperature (21 ± 4° C), humidity (40-60%), room ventilation (12.5 air changes per h) and light cycles (12 – hour light/dark cycle; on 7 A.M./off 7 P.M.).

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

Diabetic induction and characterization

The animals were randomly divided into two experimental groups: i) non-diabetic animals (CTRL); ii) streptozotocin diabetic animals (STZ). To induce diabetes, rats were injected with a single i.p. of freshly prepared streptozotocin (60mg/kg body weight; Sigma-Aldrich) in citrate buffer (0.09 M pH 4.8) as previously described (Pesaresi et al., 2010). Non-diabetic control rats received injections of citrate buffer alone. After 48h, diabetes was confirmed by tail vein blood glucose measurement using a commercial glucometer (One Touch UltraMini®, Johnson and Johnson Helathcare, USA). Values were taken in fasting condition, only the rats with feeding blood glucose above 300 mg/dl were considered diabetic. Body weight was assessed every week. After one month from the determination of hyperglycemic status, CTRL and STZ rats were sacrificed and the hippocampus, cerebral cortex and plasma were collected and sorted at -80°C until analyzed. In particular, blood sample were first collected in heparin tubes, then placed in centrifuge tubes and centrifuged at 2500 g for 15 min at 4°C to obtain plasma.

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

Assessment of neuroactive steroids

For the quantitative analysis of different neuroactive steroids, the hippocampus, cerebral cortex and plasma were extracted and purified as previously described by Caruso et al. (2010).

Materials

13C3-17β-E (2 ng/sample), C13-PROG (0.4 ng/sample) and C13-PREG (10 ng/sample), were used as internal standards, PREG, PROG, DHP, THP, ISOPREG, DHEA, T, DHT, 3α-diol, 3β-diol, 17α-E and 17β-E levels were assessed on the basis of calibration curves by high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS).

Briefly, the samples were spiked with 13C3-17β-E (2 ng/sample), C13-PROG (0.4 ng/sample) and C13 -PREG (10 ng/sample), as internal standards, and homogenized in 2 ml MeOH/acetic acid (99:1 v/v) using a tissue lyser (Qiagen, Milan, Italy). After an overnight extraction at 4°C, samples were centrifuged at 12,000 rpm for 5 min, and the pellet was extracted twice with 1 ml MeOH/ acetic acid (99:1 v/v). The organic phases were combined and evaporated to dryness. The organic residues were resuspended with 3 ml MeOH/H_2O (10:90 v/v) and passed through SPE cartridges, previously activated with MeOH (5 ml) and MeOH/H_2O 1:9 v/v (5 ml). The steroids were eluted in MeOH, concentrated and transferred in autosampler vials before liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Quantitative analysis was performed on the basis of calibration curves prepared daily and analyzed as previously described (Caruso et al., 2013b). Briefly, blank samples [6% albumin in phosphate-buffered saline (PBS)] or cortex homogenate (obtained from the control rats) were spiked with 13C3-17β-E (2 ng/sample), C13-PROG (0.4 ng/sample) and C13-PREG (10 ng/sample), as internal standards. Increasing amounts (0.05–5 ng/sample) of each steroid were added. Calibration curves were extracted and analyzed as described for the experimental samples. Positive atmospheric pressure chemical ionization (APCI+) experiments were performed with a linear ion trap MS (LTQ; ThermoElectron Co., San Jose, Calif., USA) using nitrogen as sheath, auxiliary and sweep gas. The instrument was equipped with a Surveyor LC Pump Plus and a Surveyor Autosampler Plus (ThermoElectron Co.). The MS was employed in tandem mode (MS/MS) using helium as collision gas. The LC mobile phases have been previously described (Caruso et

al., 2013b). The Hypersil Gold column (100 × 3 mm, 3 ¬m; ThermoElectron Co.) was maintained at 40° C. Peaks of the LC-MS/MS were evaluated using a Dell workstation by means of the software Excalibur®, release 2.0 SR2 (ThermoElectron Co.). The samples were analysed using the transitions previously reported (Caruso et al., 2013b).

Assessment of cholesterol and oxysterols

Materials

Cholesterol and cholesterol-2,2,3,4,4,6-d6 as internal standard were purchased from Sigma-Aldrich; 7α-hydroxycholesterol, 7β-hydroxycholesterol were purchased from Steraloids, 24(S)-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, 24-25-epoxycholesterol, 7-ketocholesterol and desmosterol were purchased from ResearchPlus.

Positive atmospheric pressure chemical ionization (APCI+) experiments were performed with a linear ion trap-mass spectrometer (LTQ, ThermoFisher Co., San Jose, CA, USA) using nitrogen as sheath, auxiliary and sweep gas. The instrument was equipped with a Surveyor liquid chromatography (LC) Pump Plus and a Surveyor Autosampler Plus (ThermoFisher Co., San Jose, CA, USA). The mass spectrometer was employed in MS/MS mode using helium as collision gas.

The LC eluents were (phase A) acetonitrile (ACN)/methanol (MeOH)/water (H_2O) (76;20;4, v/v) and (phase B) propan-2-ol(IPA). The gradient (flow rate 1 ml/min) was as follows: T0 100% A, T15 100% A, T15.50 50% A, T35 50% A, T35.50 100% A, T50 100% A. The Intersil ODS-2 150mm x 4.6mm, 5µm; (GL Sciences, Tokyo, Japan) column was maintained at 30°C.

Samples tissue obtained from hippocampi and cerebral cortex of CTRL and STZ rats, were carefully weighted, added with internals standards and homogenized in 1 ml of MeOH/ACN (1:1, v/v) using the TissueLyser (Qiagen, Italy). Samples were centrifuged at 12,000 rpm for 5 minutes and supernatant was fractionated for the analysis of free and total cholesterol and oxysterol. Acid hydrolysis was performed for quantitative determination of total cholesterol and oxysterol according to Cermenati et al. (2012). The organic residue was resuspended in 50µl of phase A and transferred in autosampler vials before the LC-MS/MS analysis.

Quantitative analysis was performed on the basis of calibration curves prepared and analysed in the same day.

Real-time Polymerase Chain Reaction

Total RNA from snap-frozen hippocampus and cerebral cortex, was extracted using the standard Trizol protocol based on the method developed by Chomczynski and Sacchi (1987). RNA was subsequently extracted in concordance with the manufacturer's protocol (Bio-Rad Laboratories, Segrate, Italy) and prepared using the Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, Calif., USA). RNA was quantified by NanoDropTM 2000 (ThermoFisher scientific, Milano, Italy). RNA was analyzed using a TaqMan quantitative real-time PCR insotrument (CFX96 real time system; Bio-Rad Laboratories, Segrate, Italy). The samples were run in 96-well formats as multiplexed reactions with a normalizing internal control, 36B4 (Eurofins MWG- Operon, Milano, Italy) and in duplicate using the iTaqTM Universal Probes One-Step Kit (Bio-Rad, Segrate, Italy). Specific TaqMan MGB probes and primers sequence were purchased from Eurofins MWG-Operon (Milano, Italy) and Life Technologies Italia (Monza, Italy). In particular, Steroidogenic acute regulatory protein (StAR), Sterol regulatory element-binding protein 2 (SREBP-2), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA R), 24-Dehydrocholesterol Reductase (DHCR24), Sterol O-acyltransferase (SOAT1), Hormone-sensitive lipase (HSL), cholesterol 24-hydroxylase (CYP46A1), ATP-Binding Cassette A1 (ABCA1), ATP-Binding Cassette G1 (ABCG1), Apolipoprotein E (ApoE), low-density lipoprotein receptor (LDLR), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and mitochondrial transcription factor A (TFAM) were obtained from Eurofins (sequences are available in table 1). Cytochrome P450 side chain cleavage (P450scc) (Rn00568733_m1), 5alpha-reductase 1 (5α-R1) (Rn00567064_m1), Superoxide dismutase 1 (SOD-1) (Rn00566938_m1), Superoxide dismutase 2 (SOD-2) (Rn00690588_g1), Uncoupling protein 2 (UCP2) (Rn01754856_m1) were purchased from Life technologies.

Mitochondrial DNA content

Total DNA from snap-frozen hippocampus and cerebral cortex was prepared by using ZR Genomic DNATM - Tissue MiniPrep kit (Zymo Research, Irvine, CA, USA). DNA was quantified by NanoDropTM 2000 (ThermoFisher scientific, Milano, Italy). DNA was analyzed using a TaqMan quantitative real-time PCR instrument (CFX96 real time system; Bio-Rad Laboratories, Segrate, Italy). Mitochondrial DNA (mt-DNA) content was analyzed using a real-time PCR

assessing mt-CoxII (cytochrome c oxidase, subunit 2) and 36B4 content as mitochondrial and nuclear encoded genes, respectively. The samples were run in 96-well formats as multiplexed reactions and in duplicate using the Bio-Rad Master mix for PCR (Bio-Rad, Segrate, Italy). 36B4 and mt-CoxII primers sequences and TaqMan MGB probe were obtained from Eurofins MWG-Operon (sequences are available in table 1).

Table 1 Eurofins MWG-Operon Primers and TaqMan MGB probes sequences

Gene	Forward primer	Reverse primer	TaqMan MGB probe
Reference gene			
36B4	GGATGACTACCCAAAATGCTTC	TGGTGTTCTTGCCCATCAG	TCTGCTTGGAGCCCACATTGTCT
Target genes			
StAR	GGCAAGGTGTTCCGACTG	GTGTCTTTTCCAATCTTCTTCAGG	AGAGTCTGTCCATGGGCTGGTCTA
SREBP-2	CTCCTTTAACCCCTTGACTTC	ACCAGCCTCCAGAACC	TGACTCCAGTGACAGTACATTGC
HMG-CoA R	TGGAGAGTGCAGAGAAAGG	GAGGGTTTCCAGTTTGTAGG	TGCGAAGTTCCTTAGTGATGC
DHCR24	AGAACTACCTGAAGACAAACCG	GAAGAGGTAGCGGAAGATGG	CCCTGAGACACTACTACCACCGACA
SOAT1	TCTCAAGAGTGGTATGCTCG	ACAAGTCCAGGTCCGTG	CCGGACATAATCCAGAAATGTAGG
HSL	CTACAGGACTATGTCACGCTAC	TTTGTAGTGTTCCCCGAAGG	TCCAGTTCACACCTGCCATCCG
CYP46A1	AGTGAAGGTCATGCTGGA	GCTGCACCCAATCCTT	TAACACCCTGGCGAAGTTCATGC
APOE	AGGAACTGACGGTACTGAT	GCACCTCTTTAGTCAGCCT	TGCCTTTACTTCAGTCATAGTGTCC
LODLR	AAGGCTGTGGGTTCCATA	GGGATCAGGCTGGTATACT	TACCTCATGGCGGTTGGTGAAGA
ABCA1	ACAATGGCATCCTCTGGT	GCAGATCTCCTAACTTCAAGATG	AGTCTCATCCCTCTGCTCGT
ABCG1	CCATTGCACTAGAACTGGTC	CACTGTCCAGGCCACT	CTCATCAAAGAACATAACAGGAGGG
PGC-1α	TGAGGAATGCACCGTAAATC	GTACAGCTCGAAGTCAGTTTC	CGGGATGATGGAGACAGCTATGGTTTC
TFAM	CACCCAGATGCAAAAGTTTCAG	CTGCTCTTTATACTTGCTCACAG	CACCTTCCACTCAGCTTTAAAATCCGC
mt-Cox II	ATTGTATTCCTCATCAGCTCC C	TGACAGCTGGGAGAATTGTTC	TCTTGGGCGTCTATTGTGCTTGTGT

Western blotting

Detection of respiratory chain complexes functional subunits (OXPHOS) was performed in a mitochondrial enriched fraction. Briefly, snap-frozen hippocampus or cerebral cortex was homogenized on ice in excess of lysis buffer (TrisHCl 10 mM, KCl 10 mM, MgCl2 0.15 mM) supplemented with protease cocktail inhibitor (Roche Diagnostic spa, Monza, Italy), with a glass-glass potter. Crude homogenate was mixed with 2M sucrose and then centrifuged at 1200xg at 4°C, twice. Supernatants were centrifuged again at 7000xg at 4°C and the pellets were suspended in suspension buffer (TrisHCl 10 mM, MgCl2 0.15 mM, Sucrose 0.25 M) supplemented with protease cocktail inhibitor. Samples were centrifuged at 9500xg at 4°C and the pellets were suspended in phosphate-buffered saline (PBS), pH 7.4 and EDTA 0.5 M pH 8, supplemented with protease cocktail inhibitor. The protein concentration of each sample was assayed relative to the bovine serum albumin standard according to the method of Bradford (Bradford, 1976). In the case of OXPHOS functional subunits, an equal amount of each sample was solubilized in 0.1% sodium decylsulphate (SDS) sample buffer, left for 5 min at 37°C, resolved on a 15% SDS-polyacrylamide gel, and electro-blotted overnight to a nitrocellulose membrane (Trans-blot; Bio-Rad, Milan, Italy). The membrane was blocked at room temperature in 10% non-fat dried milk. The filter was incubated overnight at 4° C with MitoProfile® Total OXPHOS Rodent WB antibody cocktail (Mitoscience, Abcam, Cambridge, UK) diluted in PBS buffer - 0.1% Tween 20 – 2.5% non-fat dried milk, washed for 1h and incubated for 2h with a horse anti-mouse secondary antibody conjugated to horseradish peroxidase (Cell Signaling, Leiden, The Netherlands). OXPHOS on filter were detected with the ECL method (Bio-Rad, Milan, Italy). Filter was then cut and stripped in order to detected GAPDH signal. The membrane was blocked at room temperature in 10% non-fat dried milk. Successively, the filter was incubated with a primary monoclonal GAPDH antibody (Santa Cruz Biotechnology inc., Heidelberg, Germany) as an internal control, then washed and incubated with a goat anti-rabbit horseradish peroxidase–conjugated secondary antibody (Sigma-Aldrich, Milano, Italy). Protein was detected on filter with the ECL method (Bio-Rad, Milan, Italy). ECL signals were acquired with a ChemiDoc™ XRS+ system (Bio-Rad, Milan, Italy) and analyzed with Image Lab™ software version 5.2.1 (Bio-Rad, Milan, Italy). The mean control value within a single experiment was set to 100 and all the other values were expressed as a percentage. Values of controls from different experiments were all within 10%.

Tiobarbituric Acid Reactive Substances (TBARS)

Tissue thiobarbituric acid reactive substances (TBARS) were determined as an index of reactive oxygen species (ROS) production. We used the formation of TBARS during an acid-heating reaction, which is widely ad-opted as a sensitive method for the measurement of lipid peroxidation, as previously described (Ha and Endou, 1992), with modifications. Briefly, 10 μg of hippocampus and cerebral cortex were homogenized in 400μl of lysis buffer (TrisHCl 0.1 M, pH 7.4; EDTA 1.34 mM; glutathione 0.65 mM) using a TissueLyser II (Qiagen, Hilden, Germany). 100 μl of homogenate was mixed with 600 μl of phosphoric acid 1% and 200μl of TBA 0.6%. Samples were then incubated at 95°C for 1 h and then cooled to RT, extracted with 1ml of n-butanol and then centrifuged at 4,000 g for 10 min. The supernatant was measured fluorometrically at an excitation wavelength of 532 nm and an emission wave-length of 553 nm. Quantification was done using the standard curve with malondialdehyde following similar conditions.

Statistical analysis

Student's t test was used to compare CTRL and STZ rats. A p-value of less 0.05 was considered significant. All statistical analyses were performed with GraphPad PRISM version 6 (San Diego, CA, USA).

Results

Short-term diabetes induces hyperglycemia and decreases body weight

Administration of streptozotocin (STZ) produces the expected manifestations of diabetes, including polyphagia, polydipsia and polyuria. As mentioned above, rats that displayed plasma glucose levels above 300mg/dl were considered diabetic and were included in the study. All rats treated with STZ exhibited an increase in plasma glucose levels (Table 1). We also observed a significant decrease of body weight in short-term diabetic rats when compared with non-diabetic controls

Table 1. Body weight and glucose level of non-diabetic (CTRL) and diabetic (STZ) rats

Animal	Body weight at sacrifice (g)	Blood glucose at sacrifice (mg/dl)
CTRL	426.30 ± 4.66	125.30 ± 6.04
STZ	328.50 ± 14.70 ***	604.10 ± 18.09 ***

Data are expressed as mean ± SEM. CTRL (n = 9). STZ (n = 9) animals per experimental group. Statistical analysis is performed by Unpaired Student's t test. *** $p < 0.001$

Short-term diabetes affects the levels of neuroactive steroids in the hippocampus and cerebral cortex

The levels of different neuroactive steroids were analyzed by LC-MS/MS in the hippocampus, cerebral cortex and plasma of non-diabetic and STZ rats. As reported in table 2, we observed in the hippocampus a significant reduction of PREG and its direct metabolite, PROG as well as T in STZ rats when compared with CTRL. Diabetes also affects the metabolism of PROG and T. Indeed, in the case of PROG we observe a significant decrease in the levels of THP associated with an increase of ISOPREG levels. In the case of T, the levels of its first metabolite, DHT, as well as of the further metabolite 3α-diol, were significantly reduced in the hippocampus of short-term diabetic rats. Similar situation occurs in the cerebral cortex. Indeed, the levels of PREG as well as its metabolites PROG, THP, T, DHT and 3α-diol were decreased by diabetic disease, while ISOPREG was not affected in this brain area (table 2).

Interestingly these effects were only partially observed in plasma, where a significant decrease occurred in the levels of ISOPREG, T and 3α-diol. The levels of other neuroactive steroids measured (i.e. DHP, DHEA and 3β-diol) were unaffected in hippocampus, cerebral cortex, and plasma.

Table 2. Levels of neuroactive steroids in plasma, hippocampus and cerebral cortex of non-diabetic (CTRL) and diabetic (STZ) rats

	Plasma		Hippocampus		Cerebral Cortex	
	CTRL	STZ	CTRL	STZ	CTRL	STZ
PREG	0.280 ± 0.063	0.424 ± 0.145	3.240 ± 0.576	1.111 ± 0.133 **	2.999 ± 0.630	1.000 ± 0.156 **
PROG	0.347 ± 0.091	0.277 ± 0.070	0.999 ± 0.224	0.318 ± 0.050 *	0.971 ± 0.230	0.297 ± 0.052 *
DHP	0.154 ± 0.042	0.143 ± 0.057	4.363 ± 0.571	3.736 ± 0.558	1.521 ± 0.159	1.556 ± 0.256
THP	0.142 ± 0.022	<0.100	1.104 ± 0.059	0.511 ± 0.089 ***	0.494 ± 0.072	0.196 ± 0.046 **
ISOPREG	0.876 ± 0.199	<0.100 **	0.211 ± 0.031	0.679 ± 0.114 **	<0.100	<0.100
DHEA	0.078 ± 0.021	0.066 ± 0.011	0.236 ± 0.044	0.196 ± 0.028	0.319 ± 0.080	0.240 ± 0.069
T	4.149 ± 0.543	0.950 ± 0.251 ***	4.464 ± 0.755	0.719 ± 0.134 ***	5.369 ± 0.574	0.803 ± 0.176 ***
DHT	0.090 ± 0.020	<0.050	1.001 ± 0.143	0.290 ± 0.063 ***	0.341 ± 0.058	0.123 ± 0.027 **
3α-diol	0.690 ± 0.136	0.156 ± 0.046 **	0.279 ± 0.038	0.151 ± 0.025 *	0.223 ± 0.029	0.099 ± 0.022 **
3β-diol	<0.050	0.057 ± 0.005	0.057 ± 0.004	<0.050	0.065 ± 0.009	0.068 ± 0.008

Data are expressed as pg/ml of plasma and pg/mg of tissue and are the mean ± SEM. n = 7 animals for each experimental group. Limit of quantification (LOQ) for THP and ISOPREG is 0.1 pg/mg tissue; for DHT and 3β-diol is 0.05 pg/mg tissue. Statistical analysis is performed by Unpaired Student's t-test. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$

Short-term diabetes affects gene expression of steroidogenic molecules in the hippocampus and cerebral cortex

On the basis of the decreased levels of PREG observed in the hippocampus and cerebral cortex we decided to assess the gene expression of Steroidogenic acute regulatory protein (StAR) and cytochrome P450 side chain cleavage (P450scc). As previously mentioned, StAR represents the rate limiting step in the biosynthesis of PREG (i.e. allowing the cholesterol influx into the mitochondrial compartment where steroidogenesis begins), while P450scc is the enzyme converting the cholesterol into PREG. In agreement with the levels of PREG in the hippocampus, short-term diabetes down-regulated the gene expression of StAR and P450scc (figure 1a, b). Moreover, accordingly, with the statistically significant decrease in DHT levels, the gene expression of 5α-R type 1 (i.e. the enzyme that convert T in DHT) was also down-regulated in the hippocampus of STZ rats (figure 1c).

In the cerebral cortex we also observed a down-regulation of StAR and 5α-R (figure 1d, f), however the gene expression of P450scc was unmodified.
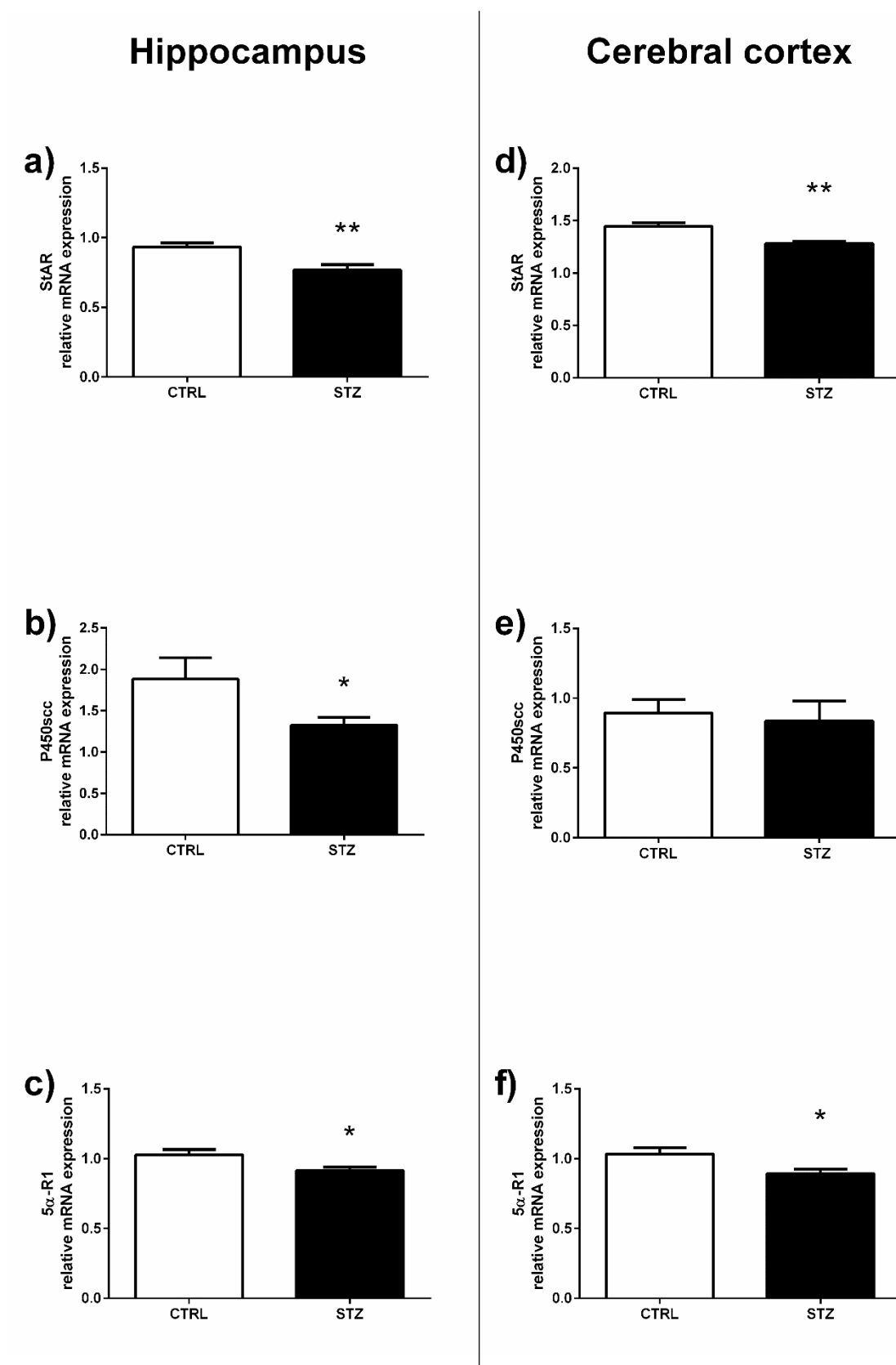
Figure 1. Short-term diabetes affects gene expression of (**a, d**) Steroidogenic acute regulatory protein (StAR), (**b, e**) cytochrome P450 side chain cleavage (P450scc), (**c, f**) 5alpha-reductase 1 (5α-R1) in the hippocampus and cerebral cortex of non-diabetic (CTRL) and diabetic (STZ) rats. Gene expression was determined by real-time polymerase chain reaction in duplicate as multiplex reactions with a normalising internal control (36B4). The columns represent the mean ± SEM, n = 7 animals for each group. Statistical analysis was performed by unpaired Student's t-test. * $p < 0.05$; ** $p < 0.01$

Short-term diabetes alters the levels of cholesterol and its metabolites in hippocampus and cerebral cortex

Cholesterol is the precursor of steroids. The levels of cholesterol and its metabolites 24(S)-hydroxycholesterol (24(S)-OH), 25-hydroxycholesterol (25-OH), 24,25-epoxycholesterol, 27-hydroxycholesterol (27-OH), 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH) and 7-ketocholesterol (7-keto) were assessed by LC-MS/MS in the hippocampus and cerebral cortex of non-diabetic and STZ rats. As reported in table 3, diabetic disease increased the level of total cholesterol, while free cholesterol was not altered in the hippocampus of diabetic rats. Assessment of cholesterol metabolites indicates a significant decrease in 24(S)-OH levels in the hippocampus of STZ rats associated with a significant increase in 7α-OH, 7β-OH and 7-keto. On the contrary, the levels of 25-OH 24,25-epoxycholesterol and 27-OH levels were unmodified (Table 3). The levels of desmosterol, the immediate precursor of cholesterol, in the hippocampus were also unaffected by short-term diabetes (Table 3).

In the cerebral cortex, the levels of free and esterified cholesterol, as well as desmosterol were not altered by diabetic disease (table 3). Similarly to what observed in the hippocampus, we found a significant decrease in 24(S)-OH levels associated with a significant increase in 7α-OH, 7β-OH and 7-keto. Differently, the levels of 27-OH, which are produced within mitochondrion, were significantly decreased in the cerebral cortex of STZ rats, while the levels of 25-OH and 24,25-epoxycholesterol remained unmodified by diabetic disease.

Table 3. Levels of cholesterol and oxysterols in the hippocampus and cerebral cortex of non-diabetic (CTRL) and diabetic (STZ) rats

	Hippocampus		Cerebral Cortex	
	CTRL	**STZ**	**CTRL**	**STZ**
Cholesterol	**µg/mg tissue**		**µg/mg tissue**	
Free cholesterol	27.40 ± 1.34	29.23 ± 1.44	34.65 ± 1.27	29.90 ± 2.23
Total cholesterol	25.65 ± 1.26	31.30 ± 1.70 *	61.65 ± 3.93	64.61 ± 5.52
Desmosterol	1.28 ± 0.08	1.24 ± 0.07	0.70 ± 0.07	0.68 ± 0.06
Oxysterols	**ng/mg tissue**		**ng/mg tissue**	
24(S)-hydroxycholesterol	90.07 ± 1.80	84.25 ± 1.96 *	132.3 ± 4.725	109.4 ± 2.96 ***
25-hydroxycholesterol	0.18 ± 0.01	0.18 ± 0.01	u.d.l.	u.d.l.
24,25-epoxycholesterol	0.22 ± 0.03	0.26 ± 0.04	0.25 ± 0.04	0.23 ± 0.04
27-hydroxycholesterol	0.28 ± 0.01	0.32 ± 0.02	0.61 ± 0.05	0.39 ± 0.04 **
7α-hydroxycholesterol	0.05 ± 0.01	0.06 ± 0.01 *	0.35 ± 0.02	0.50 ± 0.02 ***
7β-hydroxycholesterol	0.15 ± 0.01	0.21 ± 0.01 ***	0.93 ± 0.07	1.16 ± 0.04 **
7-ketocholesterol	0.24 ± 0.01	0.33 ± 0.02 ***	0.49 ± 0.02	0.73 ± 0.03 ***

Data are expressed as mean ± SEM, CTRL (n = 8), STZ (n = 9). Statistical analysis is performed by Unpaired Student's t-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Short-term diabetes alters the gene expression of molecules involved in the biosynthesis and metabolism of cholesterol

As previously mentioned in the introduction, several proteins and enzymes in the brain finely regulate cholesterol homeostasis. On this basis the gene expression of Sterol regulatory element-binding protein 2 (SREBP-2), which is involved in the promotion of cholesterol biosynthesis, binding the promoters of the 3-hydroxy-3methyl-glutaryl-coenzyme A reductase (HMG-CoA R), was assessed. In this context is important to remember that HMG-CoA R is the rate-limiting step in the biosynthesis of cholesterol converting 3-hydroxy-3methyl-glutaryl-coenzyme A in mevalonate (i.e. first step of biosynthetic way). As reported in figure 2, short-term diabetes down-regulates the expression of SREBP-2 in both hippocampus and cerebral cortex of STZ rats. In agreement whit SREBP-2 expression, the gene expression of HMG-CoA R results down-regulated by short-term diabetes (Figure 2 b, e). However, the decreased mRNA expression of HMG-CoA R does not reflect the levels of cholesterol detected in both brain areas.

We also assessed the gene expression DHCR24 (encoding for 24-dehydrocholesterol reductase), which catalyzes the conversion of desmosterol into cholesterol in the last step of cholesterol biosynthetic way. As shown in figure 2c DHCR24 was increased in the hippocampus of STZ rats. On the contrary, the gene expression of this enzyme results unaltered in the cerebral cortex of STZ rats when compared with non-diabetic control (Figure 2f).

Finally, we assessed the gene expression of sterol O-acyltransferase 1 (SOAT1) and the hormone sensitive lipase (HSL), which controls the equilibrium between free and esterified cholesterol (Brown et al., 1980, Chang et al., 2009). As reported in figure 3 the gene expression of HSL, the enzyme able to hydrolyzing cholesterol esters, was down-regulated in the hippocampus and cerebral cortex of short term diabetic rats, while that of SOAT1, the enzyme forming cholesterol esters, was unmodified (Figure 3a, c).
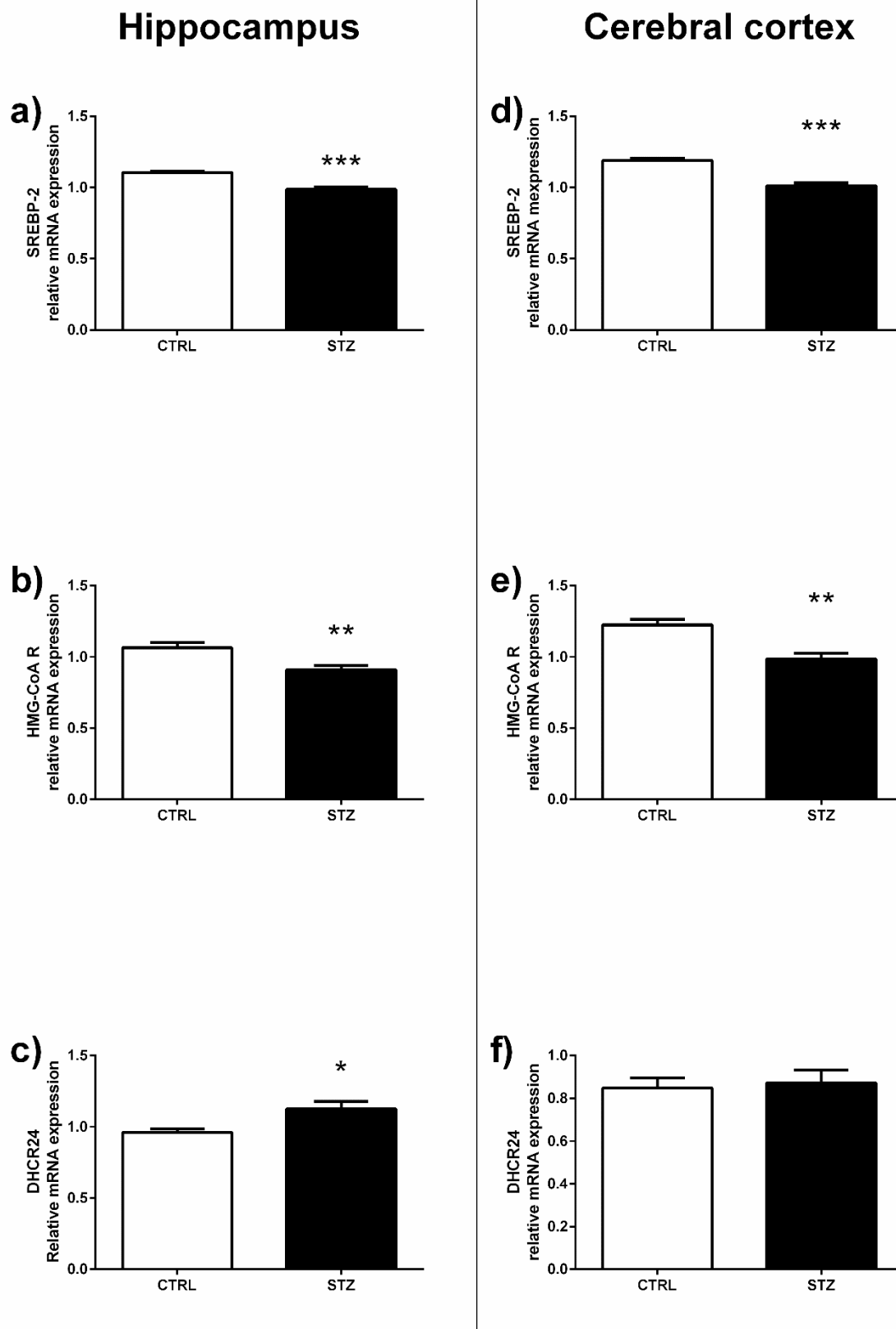
Figure 2 Short-term diabetes alters the gene expression of (**a, d**) sterol regulating element binding protein 2 (SREBP-2), (**b, e**) 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA R), (**c, f**)24-dehydrocholesterol reductase (DHCR24) in the hippocampus and cerebral cortex of non-diabetic (CTRL) and diabetic (STZ) rats. Gene expression was determined by real-time polymerase chain reaction in duplicate as multiplex reactions with a normalising internal control (36B4). The columns represent the mean ± SEM, n = 7 animals for each group. Statistical analysis was performed by unpaired Student's t-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
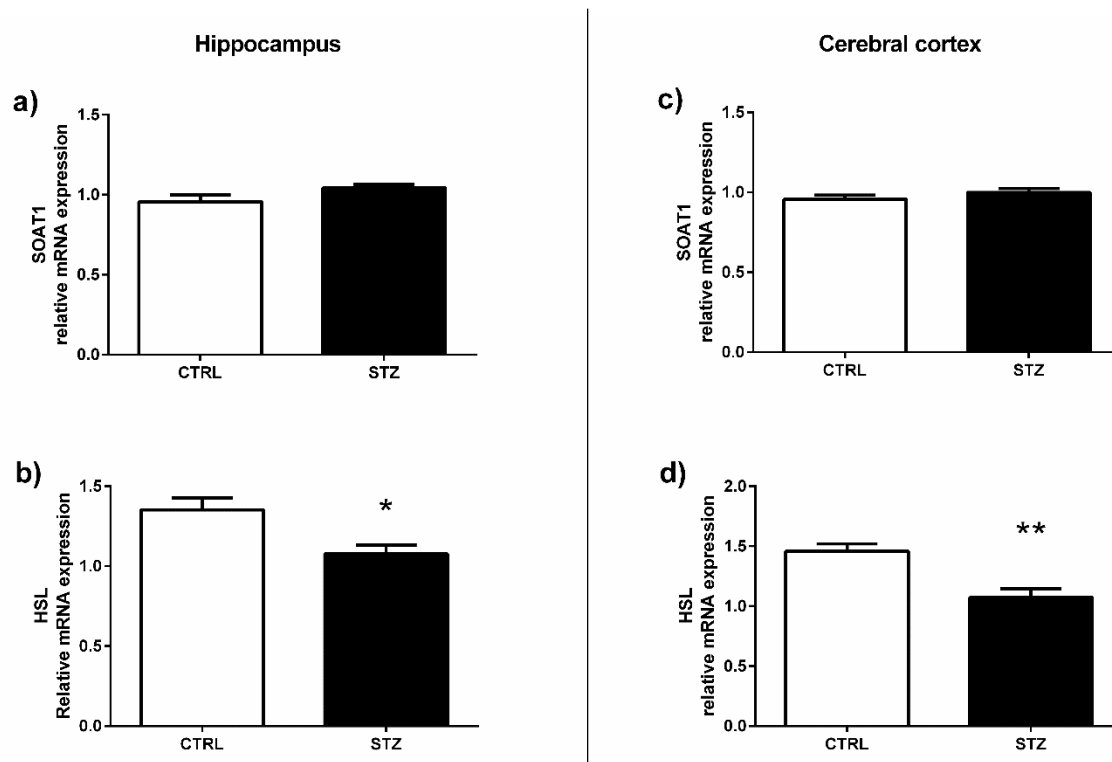
Figure 3 Short-term diabetes alters the gene expression of (**a, d**) Sterol O-acyltransferase 1 (SOAT1), (**b, e**) hormone sensitive lipase (HSL) in the hippocampus and cerebral cortex of non-diabetic (CTRL) and diabetic (STZ) rats. Gene expression was determined by real-time polymerase chain reaction in duplicate as multiplex reactions with a normalising internal control (36B4). The columns represent the mean ± SEM, n = 7 animals for each group. Statistical analysis was performed by unpaired Student's t-test. * $p < 0.05$; ** $p < 0.01$.

Short-term diabetes alters cholesterol trafficking

Cholesterol homeostasis in the CNS is composed by synergic actions of storage, metabolism, trafficking and efflux. In particular, trafficking and efflux are mediated by several molecules such as apolipoprotein E (ApoE), low density lipoprotein receptor (LDLR) and ATP-binding cassette A1 and G1 (ABCA1 and ABCG1). ApoE is the major apolipoprotein in the CNS required to exchange cholesterol between neuronal and non-neuronal cells, which binds free cholesterol for mobilization. As shown in figure 4c, the gene expression of ApoE was increased in the hippocampus, but down regulated in the cerebral cortex of STZ rats (figure 5c). On the contrary, LDLR that binds the ApoE-containing cholesterol was unchanged in the hippocampus (figure 4d) and down-regulated in the cerebral cortex of STZ rats (figure 5d) when compared with controls. ABCA1 and G1 are involved in many processes such as the regulation of lipid transport and sterol efflux. We here reported that short-term diabetes increases the gene expression of both ABCA1 and G1 (carrying cholesterol after ApoE internalization) in both hippocampus (figure 4a, b) and cerebral cortex (figure 5a, b) when compared with controls.
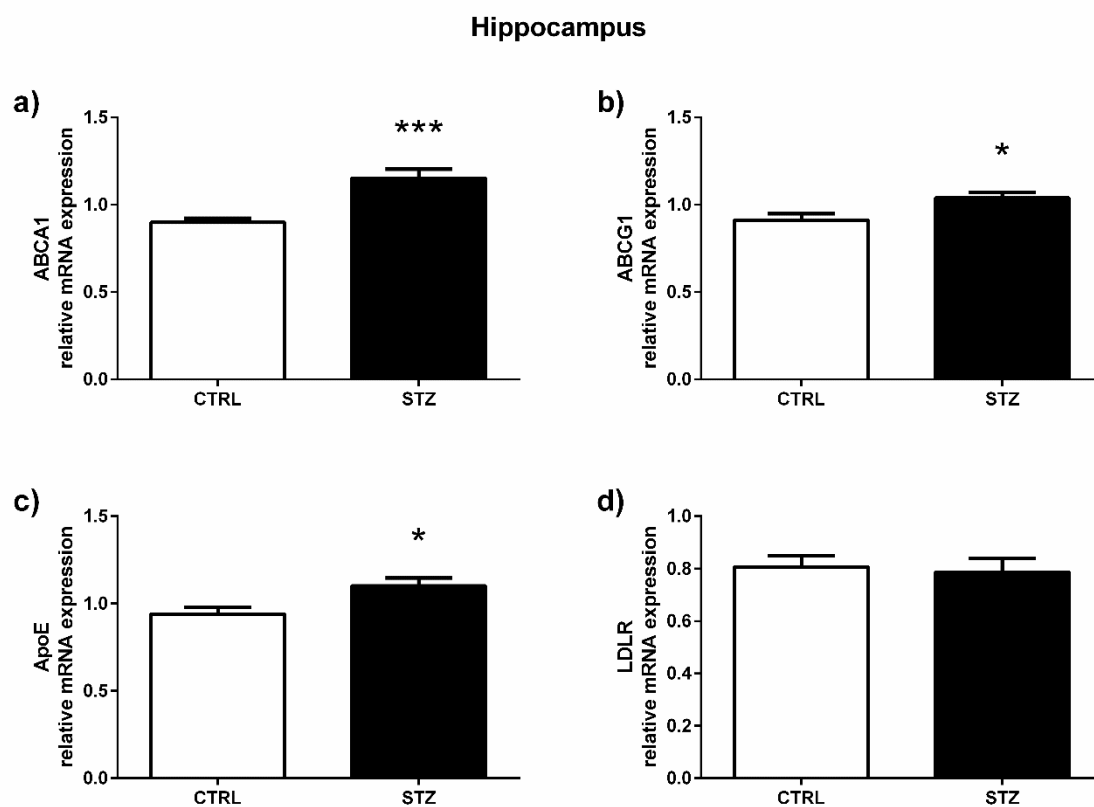
Hippocampus

Figure 4 Short-term diabetes alters the gene expression of (**a**) ATP-binding cassette (ABC) A1, (**b**) ABCG1 (**c**) Apolipoprotein e (ApoE) (**d**) Low density lipoprotein receptors (LDLR) in the hippocampus of non-diabetic (CTRL) and diabetic (STZ) rats. Gene expression was determined by real-time polymerase chain reaction in duplicate as multiplex reactions with a normalising internal control (36B4). The columns represent the mean ± SEM, n = 7 animals for each group. Statistical analysis was performed by unpaired Student's t-test. * $p < 0.05$; *** $p < 0.001$.
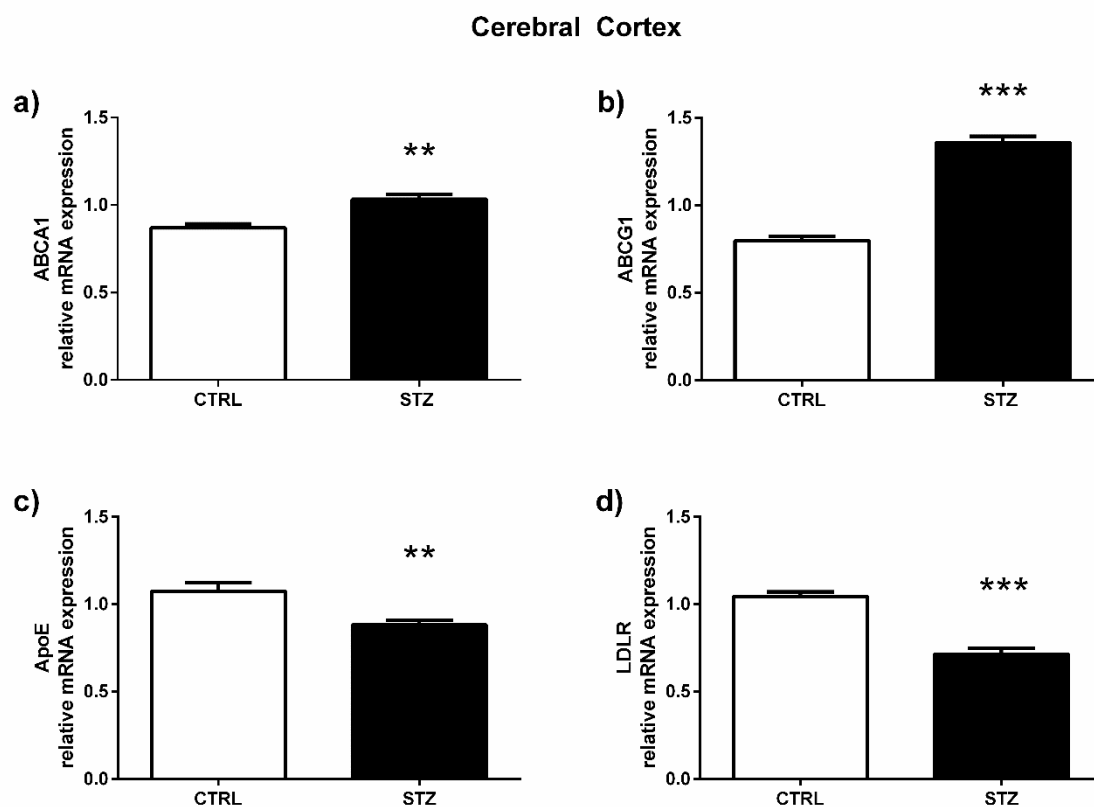
Figure 5 Short-term diabetes alters the gene expression of (**a**) ATP-binding cassette (ABC) A1, (**b**) ABCG1 (**c**) Apolipoprotein e (ApoE) (**d**) Low density lipoprotein receptors (LDLR) in the cerebral cortex of non-diabetic (CTRL) and diabetic (STZ) rats. Gene expression was determined by real-time polymerase chain reaction in duplicate as multiplex reactions with a normalising internal control (36B4). The columns represent the mean ± SEM, n = 7 animals for each group. Statistical analysis was performed by unpaired Student's t-test.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Short-term diabetes induces oxidative stress and mitochondrial alterations

Steroidogenesis is a sequence of highly compartmentalized reactions, which implies as the first step the translocation of free cholesterol from cytoplasm to the mitochondrial membrane (Giatti et al., 2012a). Moreover, hyperglycemic status increases the production of free radicals in the CNS and decreases antioxidant levels in the brain of diabetic rats (Kumar and Menon, 1993, Muriach et al., 2014). Therefore, here we assessed the TBARS production (i.e. the amount of lipid peroxidation) as well as the gene expression of uncoupling protein 2, which have a role in ROS production. In addition, we analyzed the gene expression of two molecules involved in the antioxidant defense, such as superoxide dismutase 1 (SOD-1), which exerts antioxidant function in the cytoplasm, and superoxide dismutase 2 (SOD-2), which exerts antioxidant function in the mitochondrial compartment. As reported in figure 6a, after one month of diabetes, we observed an increase of TBARS production in the hippocampus. In accordance with the data of oxidative stress, a down-regulation of UCP2, SOD-1 and SOD-2 occurs in the hippocampus of STZ rats (Figure 7a, b, c). Differently, in the cerebral cortex, we have not found any alteration of TBARS production (figure 6b) while the gene expression of antioxidant proteins such as SOD-1 and SOD-2 and of UCP2 were significantly decreased by diabetic disease after one month (Figure 7d, e, f,).

We also observed that mitochondrial functionality was affected by one month of diabetes. Indeed, the content of mitochondrial DNA (Figure 8a, c) was decreased in both hippocampus and cerebral cortex of STZ rats. Moreover, the protein contents of different subunits belonging to the respiratory chain complexes (i.e. complex I- II- III- IV), with the exception of complex V (Figure 8b), were significantly reduced in the hippocampus of short-term diabetic rats. On the contrary, in the cerebral cortex, only the complex II of the respiratory chain complexes was down regulated by diabetic disease (Figure 8d).

As previously mentioned in the introduction, peroxisome proliferator-activated receptor gamma-1 alpha (PGC-1α) has an important role in mitochondrial biogenesis and modulates the intrinsic composition of mitochondria. Moreover, PGC-1α increases the expression and acts as a coactivator for mitochondrial transcription factor A (TFAM), which is mainly responsible of the transcription and replication of mitochondrial genes from the

mitochondrial genome (Wu et al., 1999, Austin and St-Pierre, 2012). As shown in figure 9 the gene expression of PGC-1α and TFAM were up-regulated by diabetic disease in the hippocampus. Differently, in the cerebral cortex, only the up-regulation of TFAM was observed (Figure 9c, d).
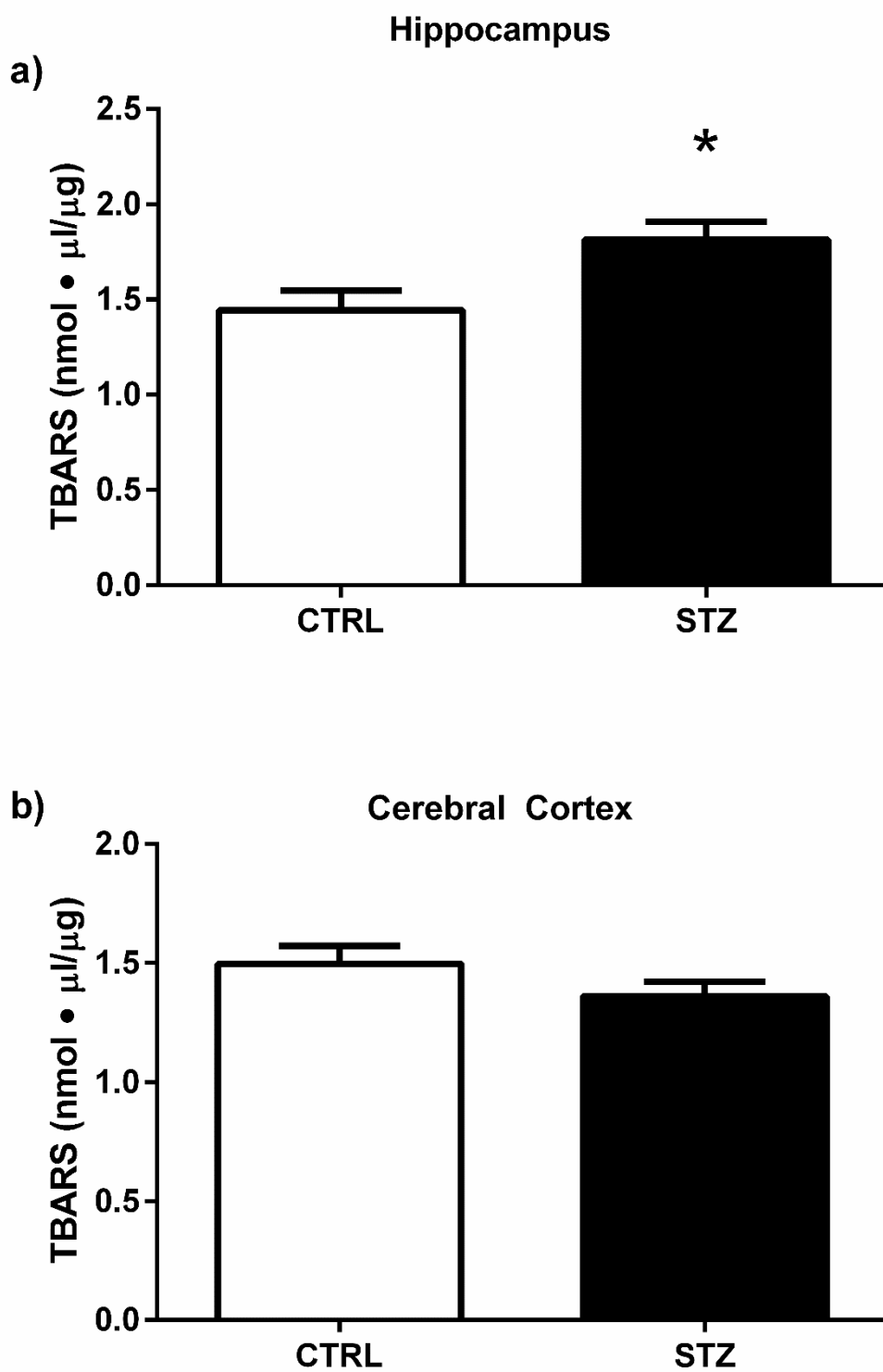
Figure 6 Assessment of lipid peroxidation (TBARS) levels in (a) hippocampus and (b) cerebral cortex of non-diabetic (CTRL) and diabetic (STZ) rats. The columns represent the mean ± SEM, n = 7 animals for each group. Statistical analysis was performed by unpaired Student's t-test. * $p < 0.05$.

Figure 7 Short-term diabetes decrease the gene expression of (**a, d**) superoxide dismutase 1 (SOD-1), (**b, e**) SOD-2, (**c, f**) uncoupling protein 2 (UCP2) in the hippocampus and cerebral cortex of non-diabetic (CTRL) and diabetic (STZ) rats. Gene expression was determined by real-time polymerase chain reaction in duplicate as multiplex reactions with a normalising internal control (36B4). The columns represent the mean ± SEM, n = 7 animals for each group. Statistical analysis was performed by unpaired Student's t-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$
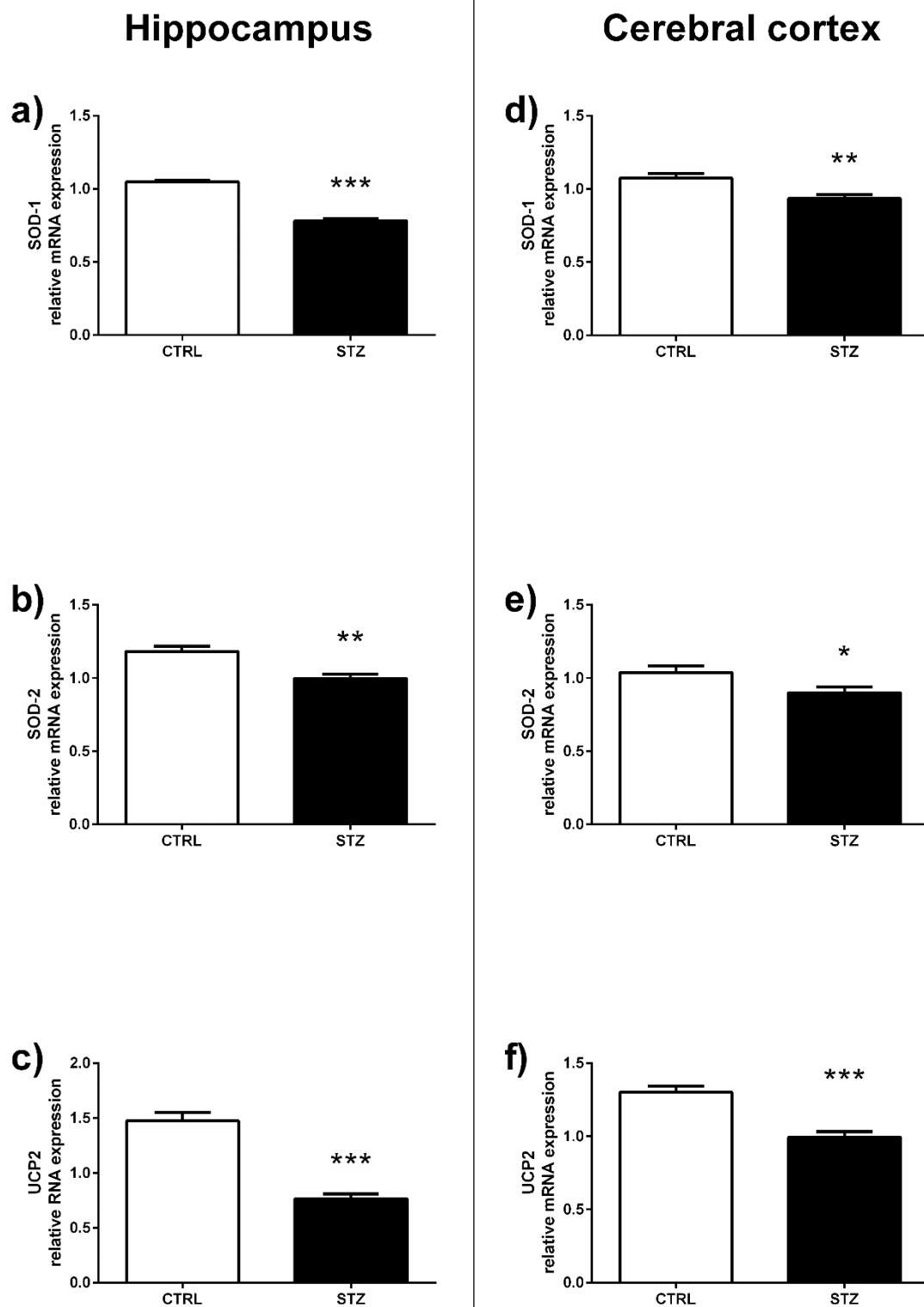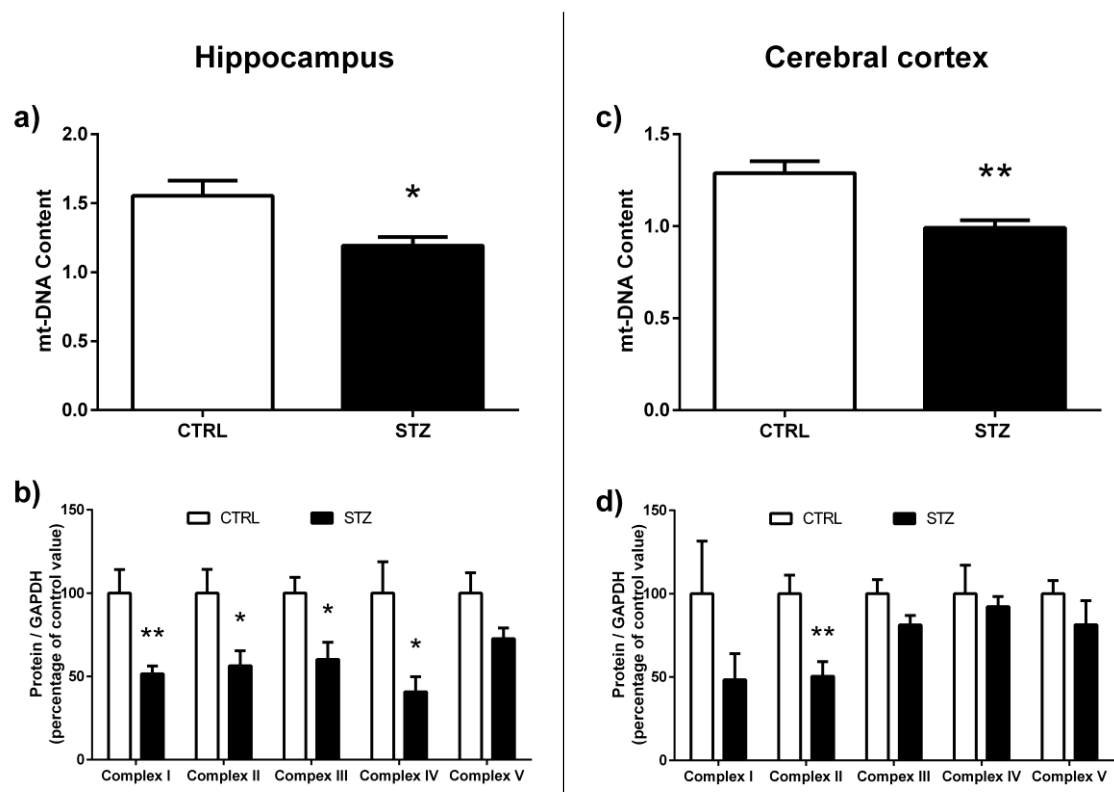
Hippocampus

Cerebral cortex

Figure 8 Short-term diabetes induces mitochondrial alterations. Assessment of (**a, c**) Mitochondrial DNA (mt-DNA) content in hippocampus and cerebral cortex of non-diabetic (CTRL) and diabetic (STZ) rats. mt-DNA was determined by real-time polymerase chain reaction of mt-Cox II (cytochrome c oxidase, subunit 2) in multiplex reactions with a reference nuclear gene (36B4). The columns represent the mean ± SEM, n = 5 animals for each group. (**b, d**) Protein content of each respiratory chain complexes (I, II, III, IV, V) in hippocampus and cerebral cortex of non-diabetic (CTRL) and diabetic (STZ) rats. Protein levels were calculated as the percentage relative to CTRL animals after normalization with GAPDH. The columns represent the mean ± SEM, CTRL (n = 6), STZ (n = 7). Statistical analysis was performed by unpaired Student's t-test. * p < 0.05; ** p < 0.01
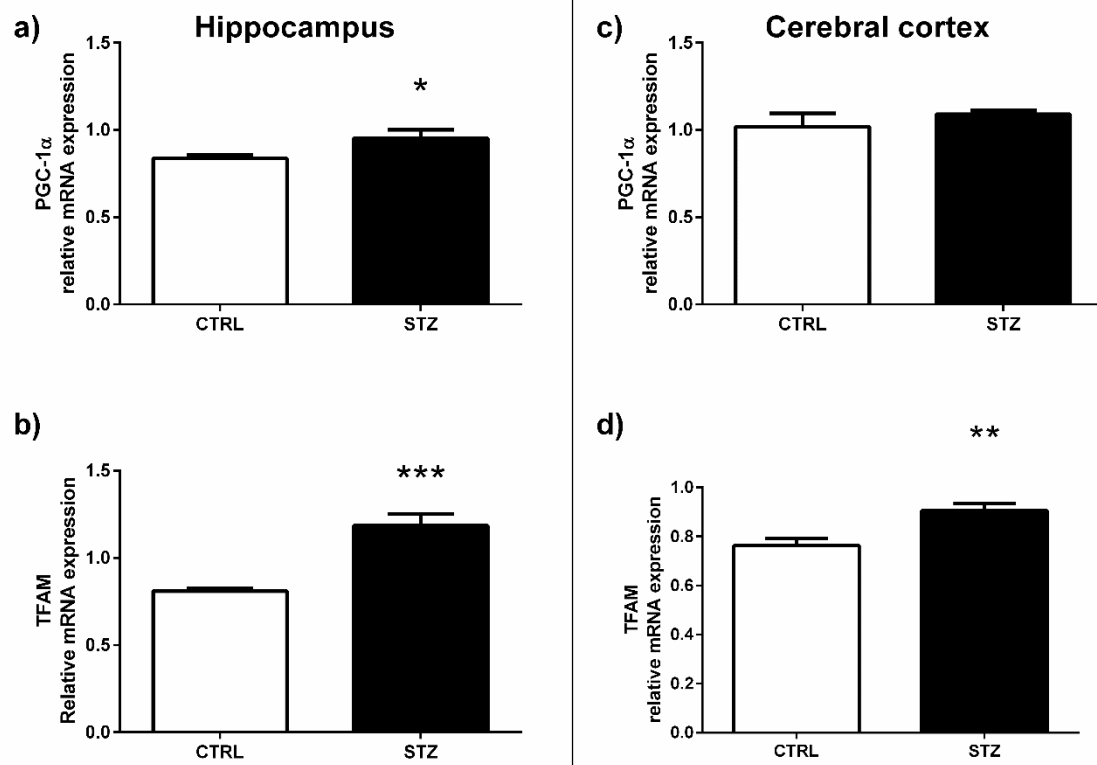
Figure 9 Short-term diabetes alters the gene expression of (**a, c**) peroxisome proliferator-activated receptor gamma-1 alpha (PGC-1α), (**b, d**) mitochondrial transcription factor A (TFAM) in the hippocampus and cerebral cortex of non-diabetic (CTRL) and diabetic (STZ) rats. Gene expression was determined by real-time polymerase chain reaction in duplicate as multiplex reactions with a normalising internal control (36B4). The columns represent the mean ± SEM, n = 7 animals for each group. Statistical analysis was performed by unpaired Student's t-test. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$

Discussion

As reported by us and other laboratories, experimental models and clinical studies show that the levels of neuroactive steroids are altered by several neurodegenerative and psychiatric disorders (Schule et al., 2014, Giatti et al., 2015a). Among these, our laboratory previously observed that in STZ rats, three months of diabetes induced altered levels of these molecules in different brain regions (Pesaresi et al., 2010). For instance, in the cerebral cortex long-term diabetes induced a significant decrease of PREG, PROG and its metabolites DHP, THP and ISOPREG as well as of T and its metabolites DHT and 3α-diol (Pesaresi et al., 2010). However, structural and functional changes in the brain may already occur after short-term diabetes (Suzuki et al., 2010, Hao et al., 2015). Accordingly, we here demonstrate that one month of diabetes (i.e. short-term diabetes) is already able to affect the neuroactive steroid levels in the hippocampus and cerebral cortex. Indeed, the levels of PREG, PROG and THP as well as of T, DHT and 3α-diol are significantly decreased both in hippocampus and cerebral cortex, while those of ISOPREG are increased only in the hippocampus. Alteration in the levels of neuroactive steroids in the nervous system could reflect altered levels of these molecules in plasma. Indeed, diabetes influences reproductive axis and sex steroids plasma levels (Babichev et al., 1998, Kim and Halter, 2014). However, differently to what we previously observed after long-term diabetes (Cermenati et al., 2010), one month of diabetes did not affect PREG levels in plasma, suggesting that early phase of diabetes directly affects steroidogenesis or steroid metabolism in the hippocampus and cerebral cortex. At least in case of the hippocampus, this is further supported by the observation that the gene expression of StAR and P450scc were decreased in the hippocampus by short-term diabetes. On the contrary, although in the cerebral cortex we observed decreased levels of PREG, only the gene expression of StAR was down-regulated by short-term diabetes after one month, while P450scc remained unchanged. This observation might suggest that decreased levels of PREG in the cerebral cortex could be due to different mechanisms compared to those occurring in the hippocampus.

Short-term diabetes also alters the steroids downstream in the biosynthetic pathway, such as T and PROG as well as the metabolism of these molecules. Indeed, we observed that the levels of 5α-reduced T metabolite, DHT, were decreased both in hippocampus and in cerebral cortex, while remains unaltered in plasma. This change was associated with a down-regulated gene

expression of the reductase enzyme (5α-R type 1) in both hippocampus and cerebral cortex. In this context, it is important to note that also PROG may be 5α-reduced into DHP (Melcangi et al., 2008). However, as here demonstrated, both in hippocampus and cerebral cortex as well as in plasma, the levels of DHP were unaffected by short-term diabetes. Three 5α-R isozymes, defined as type 1,2 and 3, have been identified in the brain (Finn et al., 2006, Traish, 2012). However, it is still unclear whether specific isozymes are responsible for the conversion of T or PROG (Finn et al., 2006, Traish, 2012).

Free cholesterol represents the substrate for steroidogenesis. Therefore, we focused our attention on whether alteration in its bioavailability may be an additional event in the effects exerted by short-term diabetes on neurosteroidogenesis. Our findings indicate that one month of diabetes significantly increases the total levels of cholesterol in the hippocampus, while in the cerebral cortex we observed only a tendency to increase. Moreover, the levels of free cholesterol were unchanged in both brain areas. As reported in literature, an increase in total levels was already observed in whole brain of STZ rats (Hao et al., 2015). The increase in total levels of cholesterol associated with unchanged levels of the free molecule may reflect an increase in cholesterol esterification. This is also suggested by the finding that, as here demonstrated, the gene expression of SOAT1 was unmodified, but the expression of HSL (i.e. the enzyme responsible for the de-esterification of cholesterol) was down-regulated in both brain areas.

In this context is important to remember that HSL play a role in steroidogenesis. Indeed, deficiency or inhibition of HSL blocks the release of cholesterol from lipids droplets, diminishes StAR expression and steroid levels. It is interesting to note that in agreement with previous findings in other experimental models (Shen et al., 2003, Kraemer et al., 2004), the decrease of HSL in the hippocampus as well as cerebral cortex of diabetic rats was associated with a decrease in StAR expression levels, suggesting a coordinated regulation of free cholesterol levels and steroidogenesis.

Although the levels of the cholesterol precursor desmosterol were unchanged in the hippocampus of diabetic animals, the expression of DHCR24, the enzyme that converts desmosterol in cholesterol, was increased. On the contrary, desmosterol and DHCR24 were unaffected by short-term diabetes in the cerebral cortex. Previous studies have shown that treatment with PREG or PROG increases the levels of desmosterol and inhibits DHCR24

expression in cultures of HepG2 cells (Lindenthal et al., 2001) and Chinese hamster ovary cells (Metherall et al., 1996). Therefore, the observed decrease of PREG and PROG levels in the hippocampus of short-term diabetic rats may be involved in the upregulation of DHCR24.

In the turnover of cholesterol, it is also extremely important the formation of oxysterols, such as 24(S)-OH, 25-OH, 27-OH, 7α-OH, 7β-OH and 7-keto. As here reported, 24(S)-OH levels were decreased in the hippocampus and cerebral cortex of STZ-rats. Interestingly, a slowed cholesterol metabolism, as observed after disruption of the mouse CYP46A1 gene (i.e., the enzyme producing 24(S)-OH), was associated with alterations in spatial, associative and motor learning, cognition, long-term potentiation and brain atrophy (Lund et al., 2003, Kotti et al., 2006, Djelti et al., 2015). Moreover, we also observed that the levels of 27-OH are also decreased in the cerebral cortex of STZ rats. It is important to remember that this metabolite is produced in the inner mitochondrial membrane (Bjorkhem et al., 1999). The decreased levels of 27-OH are in agreement with the down-regulation of StAR, which plays a fundamental role not only in steroidogenesis but also in the trafficking of cholesterol. Mitochondrial function perturbation may alter the expression of CYP27A1, which converts, into the mitochondrion, cholesterol into 27-OH (Armstrong, 2007). We also observed an increase of 7α-OH, 7β-OH and 7-keto levels in the hippocampus and cerebral cortex. Previous observations reported an increase of 7α-OH levels in plasma of STZ-rats (Wang et al., 2012). As previously mentioned in the introduction, oxysterols are produced from enzymatic or non-enzymatic oxidation of cholesterol (Smith et al., 1981, Addis, 1986). An increase of ROS may contribute to the increased levels of the oxidized form of cholesterol (i.e. which can be excreted). Indeed, their production by ROS has been associated with neurodegenerative events (Nelson and Alkon, 2005, He et al., 2006, Hascalovici et al., 2009, Kim et al., 2010). Interestingly, in liver (Saucier et al., 1989) and in primary cultures of astrocytes (Behr et al., 1991), 7β-OH and 7-keto inhibit the activity of HMG-CoA R. Therefore, the decreased gene expression of HMG-CoA R in hippocampus and cerebral cortex of STZ rats, which was also previously observed in the hypothalamus of STZ mice (Suzuki et al., 2010), could be due to the increase in 7β-OH and 7-keto levels observed in the same brain region.

As mentioned in the introduction, cholesterol homeostasis is finely regulated through different mechanisms: synthesis, transport, and elimination of excessive cholesterol from the brain (Smiljanic et al., 2013, Smiljanic et al., 2014). In particular, trafficking of cholesterol is

based on ApoE (i.e. which exchange cholesterol between neuronal and non-neuronal cells) and LDLR (i.e. involved in the uptake of cholesterol); while efflux is mediated by ABCA1 and ABCG1. We here observed in the hippocampus of STZ rat, an increase of gene expression of ApoE, ABCA1 and ABCG1, without any significant changes in that of LDLR. Probably this effect is due to the increased levels of non-enzymatic oxysterols. In this context, it is important to remember that oxysterols are potent activator of LXR and that may promote the transcription of ABCA1 as well as ApoE, two classical LXR target genes. Indeed, as demonstrated in cultures of neurons and glial cells, treatment with oxysterols induces an increase in gene expression of ABCA1 and ApoE (Fukumoto et al., 2002, Vaya and Schipper, 2007). A different situation occurs in the cerebral cortex, where ABCA1 and ABCG1 are up-regulated by diabetic disease, while ApoE and LDLR were down-regulated. These data may suggest that the observed alterations of cholesterol trafficking and efflux in the cerebral cortex are probably due to different mechanisms than those supposed for the hippocampus. In conclusion, this set of experiments suggests that the impairment of PREG observed in both hippocampus and cerebral cortex of diabetic rats is not only associated with an alteration of the steroidogenic machinery but also with altered cholesterol homeostasis.

On the other hand, due to the prominent role of mitochondria in steroidogenesisis, alteration of mitochondrial function in short-term diabetes might also contribute to the altered neurosteroidogenesis observed in the hippocampus and cerebral cortex.

Mitochondria contain their own genetic material, the mt-DNA, which encodes for essential subunits of the respiratory chain complexes. These proteins are necessary for the generation of ATP through the respiratory chain (Wallace et al., 2010). This is achieved through the electron flow through the respiratory chain that also generates ROS, which lead to oxidative stress when antioxidant defenses are insufficient (Lambert and Brand, 2009). ROS production may also affect the mitochondrial compartment in diabetic encephalopathy (Ceriello, 2003, Jing et al., 2013). In addition, it has been reported that the activity of SOD-2, a mitochondrial antioxidant molecule is impaired in the diabetic brain (Singh et al., 2004, Mastrocola et al., 2005, Cardoso et al., 2013) as well as the activity of SOD-1, which is the cytoplasmic isoform (Raza et al., 2015). Furthermore, significant increases in lipid peroxidation, mitochondrial respiratory dysfunction and compromised energy metabolism have been detected in the brain after 4 days of diabetes (Ortiz et al., 2013). Our findings reveal that oxidative stress conditions

observed in diabetic encephalopathy are associated with mitochondrial impairment. Indeed, in the hippocampus of short-term diabetic rats we observed an increase in TBARS levels, while in the cerebral cortex the levels remain unchanged.

Moreover, our findings suggest that altered gene expression of SOD-2 may be involved in the mitochondrial impairment. Indeed, we observed in both cerebral structures that SOD-1 and SOD-2 are down-regulated by diabetic disease after one month. Several studies have demonstrated roles for UCPs in the modulation of ROS production (Negre-Salvayre et al., 1997, Arsenijevic et al., 2000, Sullivan et al., 2003, Sullivan et al., 2004a, Sullivan et al., 2004b). For instance, leptin-deficient mice shown decreased levels of UCP2 and increased ROS production in macrophages (Lee et al., 1999). In agreement with these findings, we have here reported that gene expression of UCP2 is down-regulated in hippocampus of short-term diabetic rats while the levels of TBARS are increased. However, in the cerebral cortex of STZ rats, despite reduced expression of UCP2, no change in TBARS levels were observed. Collectively these data indicate that probably hippocampus is more susceptible than cerebral cortex to diabetes in terms of ROS production leading to lipid peroxidation.

To corroborate this hypothesis, we also reported a decrease in mitochondrial DNA content (a hallmark of mitochondrial dysfunction) in both hippocampus and cerebral cortex. Furthermore, we found that most of the protein belonging to each respiratory chain complexes (i.e. complex I- II- II- IV- V), were decreased in the hippocampus while in the cerebral cortex only a significant down-regulation of the complex II was detected. We here also reported that the gene expression of TFAM (i.e. the main responsible of the transcription and replication of mitochondrial genes) was up-regulated in both hippocampus and cerebral cortex of short-term diabetic rats. However, while in the hippocampus increased TFAM mRNA levels were concomitant with increased PGC-1α expression, this was not the case in cerebral cortex, indicating, once again, that the two areas considered in our study are differently affected by diabetes. Moreover, it has been reported that, PGC-1α is activated under oxidative stress in cultured skeletal myotubes, and it is required in the neuronal cells for the induction of many ROS-detoxifying proteins including SOD-2 and UCP2. Moreover, up-regulation of PGC-1α is also requested to protect neuronal cells from oxidative stress mediating cell death (St-Pierre et al., 2006). On this basis, the up-regulation of PGC-1α observed in the hippocampus

but not in cerebral cortex, might be interpreted also as a response mechanism to oxidative stress, which occurs in diabetic rats.

In conclusion, our findings indicate that short-term diabetes affects the levels of PREG, PROG, THP, T, DHT and 3α-diol in the hippocampus and cerebral cortex, while the levels of ISOPREG are affected only in the hippocampus. With the exception of T and 3α-diol levels, these changes were not observed in plasma and were associated with a decreased expression of steroidogenic molecules, such as StAR, P450scc (only in the hippocampus) and 5α-R type 1. Furthermore, we here reported that the altered levels of neuroactive steroids detected in hippocampus and cerebral cortex after short-term diabetes are probably due to a compromised cholesterol homeostasis and mitochondrial dysfunction. These latter two pathways are differently affected in hippocampus and cerebral cortex by short-term diabetes indicating that the pathology may impact the two brain areas with different molecular mechanisms.

Future experiments will be needed to investigate in further detail the alterations which may occur in the hippocampus and in the cerebral cortex, in order to deepen the cause of low levels of PREG as well as the altered cholesterol homeostasis and the mitochondrial impairment. Moreover, we will investigate the functional effects of the reduced neuroactive steroid levels in hippocampus and cerebral cortex. In particular, the expression of classic and non-classic steroid receptors as well as the neurotrophic factors will be assessed. The definition of these effects will represent the background for future experiments designed to evaluate the possible protective effects of neuroactive steroids in the diabetic encephalopathy. Indeed, as previously mentioned in the introduction, neuroactive steroids may be candidate as protective agents and therapeutic tools in several neurodegenerative diseases (Schumacher et al., 2003, Veiga et al., 2004, Garcia-Ovejero et al., 2005, Giatti et al., 2012b, Giatti et al., 2015b). Moreover, in our laboratory we have already demonstrated that DHT and 3α-diol exert analgesic effects on diabetic neuropathic pain (Calabrese et al., 2014).

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