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Role of estrogen in hypothalamic mitochondria

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Budapest, Hungary

2016

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1 Abbreviations

AF- Activating factor

AgRP- Agouti related peptide

AMPK- AMP activated kinase

AVPV- anteroventral periventricular nuclei

cAMP- Cyclic adenine monophosphate

CART- Cocaine and amphetamine regulated transcript

DNP- diiarylproprionitrile

E2- Estrogen

EGFR -epidermal growth factor receptor

ERE- E2 responsive element

ERα/ ERβ- E2 receptor α/β

ERαKO/ ERβKO mice- ERα/ ERβ knock out mice

GPER1- G-protein estrogen receptor 1

MAPK- mitogen activated protein kinases

MPTP- mitochondrial permeability transition pore

MCU- mitochondrial calcium uniporter

mtER- mitochondrial estrogen receptor

NPY- Neuropeptide Y

NRF- nuclear respiratory factor

OVX- Ovarieectomy

POMC- propriomelanocortin

PPT- propylpyrazoletriol

ROS- reactive oxygen species

RPV3- periventricular area of the third ventricle

UCP-uncoupling protein

VMH- ventromedial hypothalamus

Δψm -Mitochondrial membrane potential

2 Abstract

The hypothalamus is the master of neuroendocrine integration and plays key roles in many homeostatic systems and related functions. Being highly metabolically active the hypothalamus in reliant on the proper function of its mitochondria. Current research demonstrates that E2 can influence mitochondrial function in many ways; however, specific research regarding estrogen´s influence on mitochondrial function in the hypothalamus is scarce.

From the current literature, it can be concluded that E2 receptors are present in hypothalamic neurons and mitochondria enabling classic, slow effects on and non-classical rapid effects of E2. E2 is thus able to influence the functions of hypothalamic neurons in many ways and this review covers some of the mechanisms E2 can influence the hypothalamic neurons by modulating mitochondrial function.

E2 can increase the neuronal energy production by upregulating mitochondrial key enzymes for the glycolysis and the respiratory chain. E2 is also able to influence and thereby fine tune mitochondrial biogenesis, fission and fusion by upregulating important fission and fusion proteins. E2 plays a dual role in the formation and protection from ROS, on one hand, E2 does increase the formation of ROS and on the other hand E2 potentiate the antioxidant defence. Neuronal calcium levels are crucial for proper neuronal function and E2 is able increase both the inflow and the outflow of mitochondrial calcium and thus regulate the neuronal calcium balance. E2 is also able to influence the rate of apoptosis by preventing the collapse of the mitochondrial membrane potential and by increasing the resistance of mitochondria to calcium induced opening of the mitochondrial permeability transition pore.

The topic is scarcely researched and more specific studies are needed to further clarify the many possible effects of E2 on the hypothalamic mitochondria.

3 Introduction

The aim of this literature review is to understand the multifaceted role of E2 and its effects and possible effects on mitochondrial contribution to central metabolic pathways and on specific mitochondrial functions from the perspective of hypothalamic point of view with particular attention paid to estrogen´s role on mitochondria in hypothalamic neurons.

It is well known that the role of E2 is multifunctional and complex and that E2 is involved in a variety of homeostatic systems and affects virtually all cells of the body. It readily permeates the blood brain barrier and is also locally synthetized in the brain. Recent research demonstrates some of its less known effects on cells of different tissues. E2 influences mitochondrial functions in several ways through classical nuclear pathways, non-classical-non-nuclear pathways and through membrane located E2 receptors. E2 receptors are abundant in hypothalamus.

This review will focus on some of the specific effects E2 exerts on mitochondria. Such effects are e.g. mitochondrial biogenesis, fission, fusion, uncoupling, calcium balance, reactive oxygen species generation and membrane potential. In addition, its relevance in neurons and in particular in hypothalamic neurons is highlighted.

The hypothalamus is the major centre for neuroendocrine integration in the body and has key roles in the central regulation of several homeostatic systems and related functions such as reproduction and energy metabolism. Being highly metabolically active, the hypothalamus relies on proper functions of its mitochondria. Mitochondrial dynamics and functions are essential for the proper viability of the cell including, bioenergetics adaption and apoptosis.

Given that E2 is crucially involved in central feedback mechanisms in the hypothalamus, one can speculate that the effects of E2 on other intracellular functions in hypothalamic neurons, such as mitochondria are more pronounced than in more remote organs.

For better understanding of the complexity of E2, this review starts with an in introduction about E2, its receptors and a description of some of its functions on homeostatic systems before in detail describing some of the ways it affects mitochondrial function in hypothalamic neurons.

The topic is scarcely investigated and there is much research needed to reveal the true extent of Estrogens effect in hypothalamic neurons but from present data many interesting conclusions can be drawn and future areas needing research be identified.

4 Estrogen

Estrogens are C-18 steroid hormones produced both the male and female body. There are three major estrogens, estrone (E1), 17-β estradiol (E2) and estriol (E3). 17-β estradiol is the most potent and present in all vertebrates. Estrogens are well known as the primary female reproductive hormones crucial for the regulation of the cyclic reproductive functions and the development of secondary sexual characteristics (Cui et al, 2013). Estrogens, in particular 17-β estradiol also exert a variety of physiological effects on male reproduction and is essential for male libido, erection and spermatogenesis (Sculster et al, 2016). Increasing evidences for tissue or cell specific E2 actions and new and recently discovered function of E2 demonstrates that estrogens are not simply just sex hormones but important players in many homeostatic systems and in different cell signalling pathways and functions. E2 can be synthetized in non-reproductive tissues such as the brain, brown adipose tissue, liver, heart, bone, skin and fibroblasts consistent with the diversity of E2 actions (Nelson et al, 2001; Cui et al, 2013).

4.1 Estrogen synthesis

Estrogens are synthesized from cholesterol through series of reactions. The two most studied sites of E2 synthesis are the ovaries and the brain. In the ovaries, the theca cells synthesize androgens that are released to the granulosa cells and converted to E2 by aromatase that is released to the general circulation.

One of the many extragonadal areas for E2 synthesis is the brain. All required enzymes, including p450scc and aromatase responsible for the synthesis of estrogens from cholesterol are present in several brain areas including the hypothalamus and other areas connected with reproduction as well as in areas not primarily associated with reproduction. In these areas, estrogens are involved in cell protection, neuronal development and neuron plasticity. Cell specific production of estrogens have been demonstrated to occur in astrocytes, glial cells and neurons (Cui et al, 2013)

4.2 E2 receptors and signalling pathways

The classical genomic effects of E2 are mediated by estrogen receptor alpha and beta (ERα and ERβ), members of the large superfamily of nuclear steroid receptors. The ERs consists of six structurally and functionally different domains (A-F). The A and B N- terminal differs significantly between ERα and ERβ and regulates transcription by activating function 1 (AF-1) in a ligand- independent and cell specific way. The DNA binding domain (DBD) is similar between ERα and ERβ. The ligand binding domain (LBD) in the E terminal, shares 59% homology between ERα and ERβ and AF2 in the E terminal share 18%

ERα has at least three and ERβ at least five isoforms, the differences between the isoforms of the receptors indicates that E2 signalling and target gene regulation may be very variable. The two shorter ERαs (ERα 36 and ERα 46) isoforms lack the N-terminal harbouring AF1 domain but have the ability to heterodimerize with the full length ERα and decrease ERα activity. The shorter ERα receptor may also act as a membrane located ER be involved in rapid (non-genomic) E2 signalling by interacting with G-protein E2 receptor 1 (GPER1) genetically unrelated to ERα and ERβ and located on the cell surface. The shorter ERβ isoform differs from ERβ mainly in their ligand binding domain. ERβs unable to bind ligands and coactivators and lack transcriptional activity are able to dimerize with ERα and suppress ERα signalling. The relative distributions of ERα and ERβ in different tissues also contribute to the different effects of E2. Despite the differences among of ERα and ERβ they have the same affinity for 7β -estradiol (Vrtančnik et al, 2014).

4.3 Direct genomic signalling via ERα and ERβ

Considered as the classical E2 signalling pathways, The ligand binds to the receptor (ERα/ ERβ) in the cytoplasm and once activated, the receptors form homo or heterodimers (ERα/ ERα, ERα/ ERβ, ERβ/ ERβ) and binds to specific genome sequences named estrogen responsive elements (EREs) (figure1) in the promoter, introns or 3' untranslated region of target genes before modulating gene regulation (Heldring et al, 2007).

4.4 Indirect genomic signalling (ERE independent genomic signalling)

ERs can regulate gene transcription without the direct binding to the genome by interaction with other transcription factors such as Fos/jun, sp1, AP1 and influence the transcription of genes lacking EREs (figure1) (Heldring et al, 2007). The receptors in this case are tethered through protein-protein interactions to a transcription complex that contacts the DNA. This is also referred to as transcriptional cross talk. About one third of the genes regulated by E2 in the human body lacks own ERE (Björnström & Sjöberg, 2005). The receptor-ligand binding also triggers the recruitment of coactivators recruiting chromatin remodelling complexes, enhances recruitment of transcription factor and increase recruitment of RNA polymerase to transcribe target genes (Heldring et al, 2007). More than 60 protein coactivators have been described to interact with ERα/ERβ, this allows E2-ER complex to act as transcriptional activators (Vrtančnik et al, 2014). Following binding to DNA, the transcriptional effect of ER depends on two activating factors, AF1 and AF2. AF1 operates ligand independent and AF2 Ligand dependent. The AFs can operate alone but maximum transcription activity is obtained when they operate in synergy, these sites are areas for interaction for many co-activator proteins facilitating the interactions between the ER and the transcriptional machinery.

4.5 Ligand-independent genomic actions

Certain growth factors can activate protein-kinase cascades resulting in phosphorylation and activation of nuclear ERs at EREs (figure1) (Björnström & Sjöberg, 2005).

4.6 Indirect non-genomic signalling

E2 exerts some of its effects through ERs effects on gene expression; however other E2 effects are so rapid they cannot be dependent on the activation of RNA and transcription (Björnström & Sjöberg, 2005). These effects are termed non-genomic effects, sometimes non-transcriptional actions, non-nuclear actions, and are common for steroid hormones, including E2 (Maggi, 2011). The non-genomic effect of E2 does not depend on gene transcription or protein syntheses but rather on the interference with cytoplasmic or membrane bound regulatory proteins (figure1). In tissues generally considered as non-specific targets for steroid hormones, these effects seem to be particularly well expressed (Simoncini &Genazzani, 2003). Non-genomic signalling is often associated with membrane bound ERs (mER) such as GPER1and variants of ERα and ERβ (mERβ, mERα, mREX) (Vrtančnik et al, 2014). Membrane bound E2 receptors have been shown to be involved in the activation of tyrosine kinase and mitogen activated protein kinases (MAPKs) and activation of adenylate cyclase production hence in the production of cyclic AMP, as well as phospholipase C activation (Simoncini &Genazzani, 2003).

Authors have disagreed on however the non-genomic actions are mediated by a subpopulation consisting of certain isoforms of ER located on the plasma membrane or if the classical ERs can be located on the plasma membranes or not (Vrtančnik et al, 2014). No such structural isoforms with membrane localization sequences has been demonstrated and there is a growing consensus that the naturally occurring ERs itself can be present on the plasma membrane (Simoncini &Genazzani, 2003). Both ERα and ERβ have been found on plasma membrane as well as in other cell organelles including mitochondria and endoplasmic reticulum. The plasma membrane location of ERs may depend on the interaction with specific sites, the caveolae which are specialized membrane invaginations harbouring calveolin-1, a protein necessary for the transport of ERα to the calveolae and facilitating signal transduction by providing a location for many signalling molecules (Levin, 2009). Both ERα and ERβ has been demonstrated in the calveola (Björnström & Sjöberg, 2005), and in cells lacking calevolin-1, ERα is only found in the nucleus (Levin, 2009).

4.6.1 GPER1- G protein receptor coupled E2 receptor 1

In the mid 90's independent laboratories reported isolation of an orphan G protein coupled receptor on the membranes of various cell lines that was named GPR30, following years,

evidences that GPR30 binds and acts via E2 was published, indicating that GPR30 is involved in the rapid non-genomic signalling of E2 (Barton, 2012). GPER has also been implicated to be involved in transcriptional regulation. GPR30 was in 2007 recognized as GPER by the international union of pharmacology (Prossnitz & Barton, 2011; Barton, 2012). GPER is structurally and genetically unrelated to ERα/ ERβ. GPER has high affinity for E2 but lower than nuclear ERs. The rate of association and dissociation is however considerably faster and completed within minutes (Vrtančnik et al, 2014). Other ligands with affinity to ERs also activate GPER, naturally occurring estrogens including phytoestrogens, and synthetic estrogens known as xenosetrogens. GPER signalling occurs through transactivation of epidermal growth factor receptor (EGFR) and involves non receptor tyrosine kinase of the Src family, leading to the activation of metalloproteinases and the release of heparin binding EGF which activates EGFR and leads to activation of many signalling molecules including ERK1 and 2. GPER activation also induces cAMP production, intracellular calcium mobilization and PI3K activation, leading to indirect regulation of transcriptional activity (Prossnitz & Barton, 2011).

4.6.2 GPER in different organ systems

Studies on ERα knockout mice suggest that GPER regulates uterine proliferation independently from ERα in a process that however may involve crosstalk with ERα. Moreover, GPER are involved in the stimulation of primordial follicle formation, enhancing the uterine contractile response to oxytocin and regulates proliferative and apoptotic pathways involved in spermatogenesis (Prossnitz & Barton, 2011). GPER is abundant in renal tubules and seems regulates renal calcium influx under physiological conditions. Investigations could not detect Era-36 in the kidney which proposes the role of GPER (Barton, 2012). GPER and mER have been found throughout the nervous system and there are increasing evidences for the role of the aforementioned in addition to classical ERs contributing to the estrogenic effects in the nervous system including maintenance of homeostasis, regulation of synaptic plasticity and cognition, neuroprotection and more. GPER are involved in the mechanism for the release of GnRH. In the cardiovascular system, GPER is involved in vasodilation through stimulation of nitrous oxide release and in cardioprotection since its stimulation by G1 decreases inflammatory mediator release in reperfusion injury (Prossnitz & Barton, 2011)

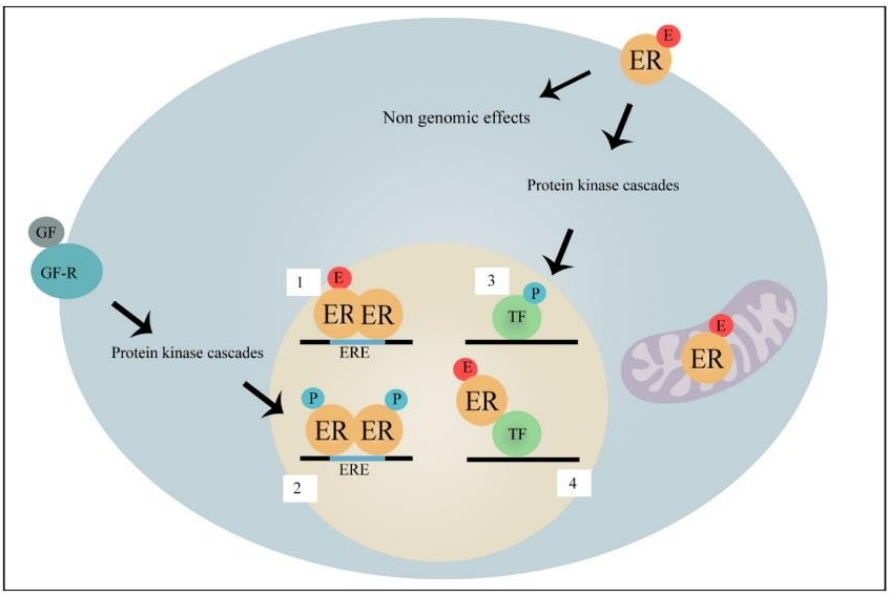

Figure 1: Illustration of the location of the E2 receptors and the four main mechanisms of E2 signalling. ERs are present in the nucleus, on the membrane and in cell organelles such as in the mitochondrial matrix. 1. Direct genomic signalling: E2 binds to and activates the ER resulting in the binding to EREs. 2. Ligand-independent genomic action: Certain growth factors (GF) are able to activate protein kinase cascades resulting in phosphorylation and activation of ERs binding to EREs. 3. Indirect non genomic signalling: E2 binds to membrane located ERs and interfere with cytoplasmic and membrane bound regulatory proteins. 4. Indirect genomic action: ERs can regulate gene transcription by interaction with other transcription factors (figure by author, 2016).

5 E2 and brain

Estrogens reach the brain from the peripheral circulation and are locally produced by neurons or astroglia. Estrogens are involved in the early development of different neuronal connections and networks irrespective of gender and in the gender specific maturation of different parts of the brain, depending on the presence or absence of E2 in the brain during specific times in the early development.

Later in life, aside from the well-known regulation of reproduction and the central involvement in circadian rhythm and food intake regulation, estrogens are involved in a variety of local processes in the brain, such as cognitive and motor behaviour associated with neuronal plasticity and neuroprotection under acute, ischemic or hypoxic injury or chronic neurodegenerative conditions. To enable this, the brain is equipped with all known ERs in varying ratio and location and expresses all enzymes necessary for the novo synthesis of E2. This allows estrogens to act in a precisely controlled manner on target cells and tissues (Arnold et al, 2012).

5.1 E2 receptors in the hypothalamus

ERs are abundantly expressed in the brain, Including the ventrolateral part, the ventromedial hypothalamus (VL VMH), the arcuate nucleus (ARC), the medial preoptic area (MPOA) and the paraventricular nucleus (PVN) (López & Tena-Sempere, 2015). Both ERα and ERβ are expressed in all hypothalamic nuclei, although the proportion of ERα is significantly higher (Foryst-Ludvid & Kintscher, 2010; López & Tena-Sempere, 2015).

5.2 Mitochondrial function in the hypothalamus

Mitochondria are cell organelles found in almost all eukaryote cells most known as the powerhouses of the cells due to their energy production. Besides their predominant role in energy metabolism by being the location for the citric acid cycle, the respiratory chain, pentose phosphate pathway and beta oxidation of fatty acids, they are involved in many other cellular processes including calcium homeostasis, reactive oxygen species (ROS) production, steroid biosynthesis and control of apoptotic signalling (Morava & Kozicz, 2013).

Brain tissue is unique in its high energy demand; the human brain constitutes approximately 2 % of the body mass but uses up to 25% of the body's fuel from mitochondrial energy production, thus, reliant on adequate mitochondrial function. E2 has been shown to influence all mitochondrial bioenergetics pathways as well as other mitochondrial functions such as calcium homeostasis, uncoupling, membrane permeability and ROS *etc.* (Morava & Kozicz, 2013).

5.3 Mitochondrial E2 receptors

The extranuclear location of ERs is now firmly established and several publications establishes evidences for mitochondrial E2 receptors (mtERs) emphasizing the role of E2 in the regulation of cellular bioenergetics (Arnold et al, 2012; Rettberg et al,2014). Already 20 years ago, an *in vitro* experiment showed that ERα and ERβ bind directly to mitochondrial DNA through mitochondrial EREs. More recent studies have demonstrated that ERβs localizes to mitochondria. Considering the predominant ERβ population in brain mitochondria it is reasonable to postulate that E2 regulates mitochondrial function partly via ERβ mediated transcription of mitochondrial DNA, however the ways of coordination between the ERs, the nucleus and the mitochondria remains to be determined and the possibility of non-genomic signalling pathways have to be considered (Rettberg et al,2014). Important questions that remain to be answered are however ERs in the mitochondria are stationary or freely moving, if mobile then how are they transported

through cellular compartments? Does other ER mediated intra mitochondrial actions occur? Besides classical genomic E2 action one have to take into account the possibility of non-genomic E2 action on mitochondria through mtERs or other mitochondrial structures/ proteins as binding sites for E2 or activated ERs (Arnold et al, 2012).

As early as 1989 Grossman et al reported findings of an ERE in the matrix of pancreatic acinar cells and a decade later it was shown that a radioactively labelled, bovine serum albumin coupled E2 binds directly to, and inhibited a subunit of the proton FOF1 mitochondrial ATPase/ ATP synthases in brain tissue, one assumed way of rapid E2 action modulating the ATP synthesis. It has also been showed that E2 rapidly inhibited Na^+ dependent Ca^{2+} efflux from mitochondria in nerve terminal, hence affecting the calcium homeostasis in nerve terminals. These data and many other similar make reasonably sure that E2 regulates mitochondria in many other ways than through the classical genomic pathway, using classical ERs on other locations as well as non-genomic ways of signalling.

6 E2 as a crucial regulator in certain feedback mechanisms

The hypothalamus is the major centre for neuroendocrine integration and have key roles in the central regulation of several homeostatic systems and related functions. Two very important homeostatic systems centrally regulated by E2 in the hypothalamus are the reproduction and central energy metabolism. These two systems will be further discussed in the following subchapters to highlight the major and well-known functions of E2 in hypothalamus.

6.1 E2 and reproduction

The hypothalamic pituitary gonadal axis (HPGA) is the key neuroendocrine pathway for reproduction. Gonadotropin releasing hormone (GnRH), a trophic peptide hormone, constitutes the initial step in the HPGA and is of major importance for reproductive functions in all mammalian species. The cell bodies of the GnRH neurons are scattered in the basal forebrain from the medial septum through the medial preoptic area to the basal hypothalamus. Although the cell bodies are widely distributed, the majority of the GnRH neurons axons projects to a highly circumscribed area within the hypothalamus, the median eminence, from where GnRH is released into the pituitary portal system allowing GnRH to travel through the bloodstream, bind to GnRH receptors on gonadotrophic cells in the anterior pituitary and drive the synthesis and release of follicle stimulating hormone and luteinizing hormone (Herbison, 2008, Kwakowsky et al, 2014). Ovarian steroid secretion in turn exerts negative and positive feedback on the GnRH neurons modulating their secretion. During the majority of the estrus cycle, E2 is responsible for the inhibition of gonadotrophin secretion by suppressing the GnRH neurons and the pituitary gonadotrophs (Gliderwell-Kenney et al, 2007). In the middle of the cycle the GnRH neurons switches from negative to positive feedback switching from a pulsatile GnRH release to a sustained high level of secretion, the so called pre-ovulatory GnRH surge. The exact mechanisms how E2 regulates the GnRH neurons are still not completely elucidated (Kwakowsky et al, 2014). In vitro and in vivo experiments on rat hypothalamus has shown that inhibitory and stimulatory effects of E2 seem to involve different hypothalamic areas which may indicate that the inhibitory and stimulatory pathways are separated, at least partly. In these experiments, the negative feedback was localized to the arcuate nucleus and the median eminence and the positive feedback was mapped to the preoptic and the suprachiasmatic nucleus (Radovick et al, 2012).

Originally the genomic actions of E2 on ERα/ERβ was considered as the primary mechanism for the E2 regulation of GnRH, however it has become clear that many other non-genomic E2 actions and additional cell signalling pathways are involved (Kwakowsky et al, 2014).

Early studies failed to demonstrate the presence of ERs in GnRH neurons and the effects of E2 where thought to occur only through interneuron networks since neurons producing kisspeptin, NPY, GABA, Glutamate and norepinephrine do present ERα and studies on rodents have demonstrated changes in the GnRH, LH and FSH secretions. However more recent studies have shown the presence of ERβ, GPER, ERx and a membrane bound ER sensitive to the diphenylacrylamide compound STX on GnRH neurons in the hypothalamus of several species indicating direct E2 action on GnRH neurons, however not through ERα (Terasawa & Kenealy, 2012). Nevertheless, Since GnRH neurons doesn't express ERα, which are demonstrated to be both necessary and sufficient for the positive feedback, the role of the ERα expressing aforementioned neurons groups, projecting upon the GnRH neurons seems to be crucial (Herbison, 2008).

In rodents, the positive E2 feedback is regulated largely by ERα expressing neurons located largely in the anteroventral periventricular nuclei (AVPV), median preoptic, ventral preoptic nuclei and periventricular preoptic nuclei in the rostral hypothalamus. Together these afferent E2 sensitive neurons form a periventricular identity that can be referred to as rostral periventricular area of the third ventricle (RPV3). The RPV3 neurons are a highly heterogeneous population found to express a variety of neuropeptide, including the ERα glutamate, GABA, Kisspeptin and neurotensin that all seems to be involved in the positive E2 feedback on GnRH neurons (Herbison, 2008). Lesion studies in rats have demonstrated the key importance of the AVPV nuclei, Lesions in the AVPV induced increased GnRH secretion, no LH surge, persistent estrus and decreased Kiss1 mRNA expression (Donato et al, 2013, Herbison, 2008), There are also evidences for ERα and ERβ expression within the neurons of the AVPV and studies have shown that AVPV neurons project upon GnRH neurons. Together these results indicate that E2 influences GnRH neurons via intermediary AVPV neurons (Herbison, 2008).

The location and the identity of the ERα expressing neurons responsible for the negative feedback is less clear and may involve several interneuron connections as well as direct genomic and non-genomic estrogenic action (Kwakowsky et al, 2014). However, most E2 mediated negative feedback regulation of GnRH secretion is mediated by neurons located in the ARC and it has been hypothesised that, as in the case of positive feedback, kisspeptin is an important mediator but in this case as a suppressor of GnRH. Dubois et al, (2015) tested this hypothesis and suggests based on their own results and previous data that although under normal circumstances, ERα signalling in kisspeptin might be enough for E2 mediated negative feedback but that E2 primarily exerts its negative feedback in the arcuate nucleus through pathways independent from kisspeptin neurons. Both GABA and POMC expression neurons have been suggested as possible mediators.

In summary, the feedback mechanisms of E2 on GnRH are highly complex and involve multiple neurohormonal pathways, interneuron connections and different types of E2 signaling.

6.2 E2 and energy metabolism

E2 is an important player in the in the regulation of many metabolic pathways including feed intake, energy expenditure, glucose and lipid metabolism on central and peripheral levels (López & Tena-Sempere, 2015). E2 treatment has been shown to prevent obesity, the prevalence of obesity increases after menopause and ovariectomy in rodents leads to increased fat accumulation. Mice of both sexes, lacking CYP 19, develop obesity in the absence of hyperphagia.

Estrogens effects on energy homeostasis seem to be largely mediated by ERα. Experiments with selective ERα and ERβ agonists showed anorectic effect by ERα agonist but not by ERβ agonists. Supporting this theory ERα knock out mice (ERαKO) displayed hyperphagia, increased adipositas, insulin resistance and hyperleptinemia (Hevener et al, 2015). In addition to ERα mediated effects, non-genomic effects of E2 cannot be excluded.

Central regulation of energy balance is mediated via complex signalling pathways integrating endocrine signalling from the periphery.

The central nervous system is made aware of the metabolic status of the body by receiving information from various metabolic pathways through signals released from peripheral tissues

indicating levels of energy storage and expenditure (Barros & Gustafsson, 2011; Frank et al, 2014).

The hypothalamus is the major brain centres for the regulation of energy homeostasis and food intake. The arcuate nuclei, a cluster of neuronal bodies with more or less similar connections and functions is regarded as the master hypothalamic centre for feeding control. The arcuate nucleus harbours two distinct neuronal cell populations regulating appetite, one expressing the neuropeptides agouti related peptide (AgRP) and neuoropeptide Y (NPY) which both are orexigenic and one population of neurons expressing the anorexigenic products of proopiomelanocortin (POMC) and the cocaine and amphetamine regulated transcript (CART). The former has been named the hunger centre and the latter the satiety centre. The hunger and satiety centre responds to peripheral nutrients including leptin, ghrelin, adiponectin, insulin and ovarian steroids (Frank et al, 2014).

ERα is considered as the major player in the central control of energy balance by E2 supported by aforementioned experiments with selective ERα/ERβ agonists and ERαKO mice (Hevener et al, 2015). Weather ERα mediates its effect via regulation of food intake or energy expenditure has been discussed controversially. The injection of small hairpin RNA- mediated ERα genes silencing ERα in the VMH in mice induced weight loss, reduction of energy expenditure, decreased thermogenic response to feeding (Foryst-Ludvid & Kintscher, 2010; Hevener et al, 2015). However, food intake was not altered indicating that E2 in the VMH primarily affects energy expenditure (Foryst-Ludvid & Kintscher, 2010). POMC expression fluctuates during the estrus cycle reaching its peak concentration during proestrus coinciding with the maximum E2 levels, after OVX in mice, the levels of POMC mRNA are reduced and restored after E2 treatment. Also in ERαKO mice the POMC mRNA levels are reduced. Specific deletion of ERα in POMC neurons in mice induced hyperphagia without direct influences on energy expenditure or fat distribution (Hevener et al, 2015). The exact molecular mechanism of E2 action on POMC neurons is not clear. Recent data showed that E2 inhibited AMP activated kinase (AMPK) in the hypothalamus and that the activation of AMPK within the ARC reversed the anorexigenic activity of E2. However, this data does not demonstrate a direct relationship between AMPK and POMC neurons (Foryst-Ludvid & Kintscher, 2010).

An alternative model suggests that E2 undermines the interaction between POMC, NPY and the melanin- concentrating hormone MCH in the cells of LHA resulting in anorexia and weight loss. E2 also triggers an increase in the excitatory inputs to POMC neurons through a signal transducer and transcription 3 activator (STAT3)- dependent mechanism. Despite the fact that many cells in several hypothalamic nuclei co-express leptin receptor, the estrogenic effects (decreased food intake and increased energy expenditure, leading to weight loss) on STAT3 are leptin independent since the effects was also seen in leptin deficient and lepRb-deficient mice. In STAT3-KO mice, the effects were not seen. This suggests that the central effects of E2 on energy metabolism are independent from leptin/leptin receptor pathways.

In vitro, E2 has been shown to activate STAT3 through signalling pathways including MAPK, Src kinse and PI3K. *In vivo* E2 activates STAT3 in less than 30 min in a fashion that may involve the Src kinase pathway. E2 does also influence the AgRP/NPY neurons but the exact mechanisms are not completely clear. In vitro studies have shown different responses to E2 depending on the relative distribution of ERα and ERβ. The greater proportion of ERα, the more suppression of NPY expression and the greater the proportion of ERβ the more NPY was expressed. However, *in vivo* results shows increased NPY levels after OVX in rats that could be corrected with E2 administration (López & Tena-Sempere, 2015). To add further complexity to the story, Olofsson et al, (2009) found no expression of ERα in AgRP and NPY neurons in hypothalamic suggesting indirect E2 regulation of these neurons through presynaptic neurons. The study also indicated the essence of AgRP and NPY neurons as mediator of Estrogens anorexic effects. They concluded that E2 rapidly controls membrane excitability through non-genomic mechanisms in the hypothalamic neurons that control feeding, that E2 decreases GABAergic inhibition of POMC neurons via a Gq coupled membrane E2 receptor and that Estrogens GABAergic inhibition of NPY/AgRP neurons increases or decreases weather it binds to ERα or Gq-mER respectively (Olofsson et al, 2009; Donato et al, 2013).

Given that E2 is crucially involved in these central feedback mechanisms centred in the hypothalamus it is reasonable to hypothesize that E2 also has an impact on other intracellular functions in hypothalamic neurons, such as mitochondria.

7 Brain glucose metabolism

7.1 E2 and glucose transport

Glucose enter the brain via GLUT transporters (integral membrane proteins). GLUT 1 has two isoforms, one responsible for the glucose transport through the blood brain barrier endothelial cells and one regulating transport from brain to glia. GLUT1 has low affinity but is highly sensitive to fluctuating glucose levels and its expression increases rapidly in case of hypoglycaemia. GLUT 3 and GLUT 4 are expressed on neuronal cells and are responsible for the glucose transport into neurons, GLUT 4 being unique because its translocation from cytosol to cell membrane is influenced by insulin. Studies in rodents and non-human primates have shown that OVX decreased GLUT 1,3 and 4 expressions and that E2 treatment prevented this decrease. Ding et al (2013) has demonstrated that with increase in age the brain's ability to utilize glucose decreased, a change that may be due to a decrease in ovarian hormones (Rettberg et al,2014)

7.2 E2 and mitochondrial glucose metabolism

Many signalling pathways regulated by E2 converges upon mitochondria and E2 facilitates the utilization of glucose trough the glycolysis, the TCA and the respiratory chain (Figure 2) *In vivo* studies in rats have shown a significant increase in the neural expression of hexokinase, phosphofructokinase and pyruvate kinase within 4 hours after E2 administration (Kostanyan & Nazaryan, 1992). Nilsen et al. (2007) conducted proteomic studies of rat brain mitochondria preceded by a single E2 injection 24 h before the experiments were conducted. The results were that out of 499 proteins investigated, 28 proteins demonstrated a two-fold or greater increase after E2 injection; several of the proteins with increased expression were key metabolic enzymes such as pyruvate dehydrogenase, aconitase and ATP synthase, pyruvate dehydrogenase being particularly interesting because of its role as the major connecting enzyme between the glycolysis and the TCA. To confirm that the measurable increase in proteins corresponded to functional changes, they conducted experiments determining the impact of E2 on the enzymatic activity if the electron transport chain. The experiment showed increase in expression and activity of complexes I, IV and V/ATP synthase F1 subunit α and β (Nilsen et al, 2007). Thus, E2 is having an enhancing effect on many important enzymes in the mitochondrion causing optimal glucose utilization in the brain (Rettberg et al, 2014).

Nilsen et al, (2007) investigated the role of ERα and ERβ in the promotion of mitochondrial bioenergetics by using the ERα selective agonist propylpyrazoletriol (PPT) and the ERβ selective agonist diarylproprionitrile (DNP) and probing the effects of signalling cascades activated through the ERs. In the experiment, hippocampal mitochondria were used. The results showed increases in the protein expression of pyruvate dehydrogenase subunit E1 and ATP synthase F1 subunit after PPT treatment. DNP treatment upregulated the expression of cytochrome oxidase subunit I, which is mtDNA encoded, PPT did not upregulate this subunit which leads to the suggestion that that mtDNA transcription is primarily regulated by an ERβ dependent mechanism. Cytochrome oxidase subunit IV is encoded by nuclear DNA and where upregulated by both PPT and DNP, suggesting that ERα and ERβ are both capable of independent upregulation of certain mitochondrial proteins. Assessment of mitochondrial respiration from in the same samples showed that the upregulation of mitochondrial proteins by PPT and DPN leads to an enhanced mitochondrial respiration (Rettberg et al, 2014).

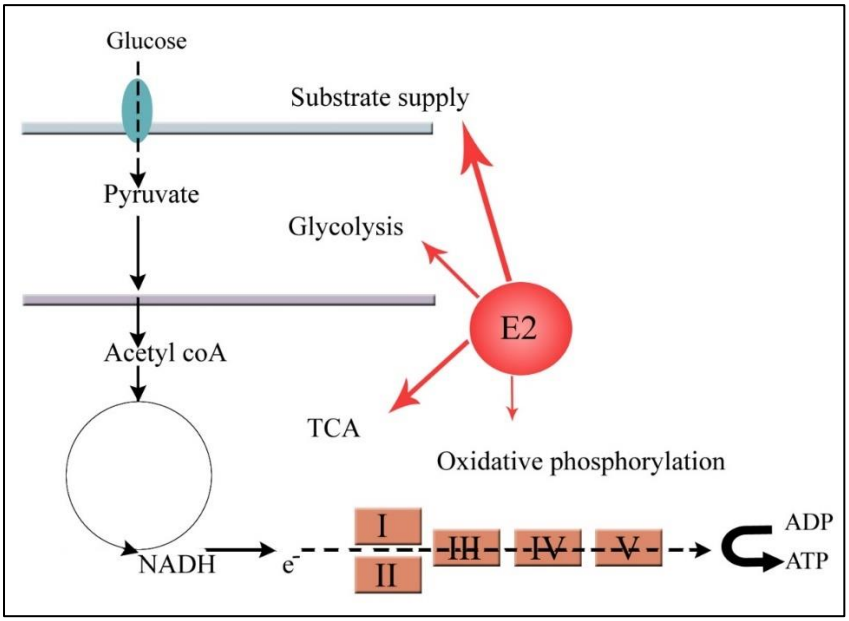

Figure 2 Illustrates E2s regulation of the cellular bioenergetics. E2 is able to upregulate key enzymes of the cellular energy generating biochemical pathways (Figure by author, 2016)

The results presented in this chapter clearly demonstrate that E2 can increase the energy production in mitochondria However; these results are obtained from brain mitochondria, not specifically from hypothalamic mitochondria. It is reasonable to believe that the results would be

true for hypothalamic mitochondria but more specific studies using hypothalamic mitochondria are needed for further verification.

8 Mitochondrial dynamics and E2

Mitochondria are highly dynamic organelles, constantly moving and with the ability to fuse (fusion) and divide (fission). Mitochondrial dynamics are essential for the maintenance, mobility, distribution and function of mitochondria and for the opposite, the programmed cell death, apoptosis.

Mitochondrial mobility modulates the intracellular distribution of mitochondria and particularly ensures the higher density of mitochondria in areas requiring a higher ATP production, such as axons and synapses. The movement of mitochondria is due to interactions with the actin cytoskeletons and microtubule to ensure their distribution and movement along axons and dendrites. For long transport, mitochondria bind via mitochondrial Miro1/2, belonging to the GTPase family, associates with Milton/TRAK and binds to kinesin and dynein motor proteins forming the mitochondrial motor/adaptor unit for their transport along the microtubule. Shorter transports are mediated by myosin motors and actin cytoskeleton. Mitochondria can anchor and form stationary pools via dynamic anchoring interactions with synapthilin, microtubules and via actin based anchoring receptors (Bertholet et al, 2016).

8.1 Mitochondrial fission, fusion and E2

Under normal conditions, the coordinated fusion and fission of the inner and outer mitochondrial membranes in response to physiological stress are in a dynamic equilibrium. When cells experience metabolic stress, the fusion and fission of mitochondria helps to maintain functional mitochondria thus the ultrastructure of mitochondria mirrors perfectly the physiological status of the cell. Moreover, mutations in the proteins involved in the fusion and fission process can result in dramatic neuropathological consequences, certain neuropathies, cause nerve atrophy, promote apoptotic processes and reduce the general survival of the cell. Fusion is mainly regulated by a large family of mitochondrial GTPases mitofusin 1 and 2 (Mfn 1 and 2) and for fission the outer membrane homolog fission 1 (Fis1) and dynamin related protein 1 (Dyn1) are essential. Neurons in particular require a very sensitive balance of fission and fusion and Dyn1 overexpression positively affects the affects the density of dendritic spines, synapses and the dendritic

localization of mitochondria that possibly could be associated with neuronal degeneration (Bertholet et al, 2016).

The ratio of fusion and fission seems to be influenced by E2. Experiments conducted in rodents have shown that in OVX rodents there is an increase in mitochondrial fission (significant increase in fission protein drp1) and a decline in mitochondrial fusion (decrease in OPA1) suggesting increased mitochondrial fragmentation corresponding to a decrease in ovarian hormones. E2 treatment prevented aforementioned changes (Arnold et al, 2012). In a study conducted by Arnold et al, (2012) where the effects of short-term E2 application on the expression of fusion and fission protein in astrocyte cultures showed a massive upregulation of all fusion and fission proteins except Mn1 after short-term (24h) E2 treatment. They also found that the specific ER antagonist ICI182780 didn't prevent this effect. By relating the effects of E2 on gene expression levels with cell proliferation and apoptotic markers in these cell cultures, they found that in only female derived astrocytes cell proliferation and viability was increased. Because those proteins are nuclear encoded they concluded that the E2 effects were indirect.

Taken together, there are convincing evidences for the effect of E2 on the fine tuning on mitochondrial dynamics but the lack of studies on hypothalamic mitochondria makes firm conclusion difficult and further studies are needed.

8.2 Mitochondrial biogenesis

Mitochondrial biogenesis is the increase in number and size of mitochondria. Owning to their bacterial origin, mitochondria have their own genome and are able to auto-replicate. It is well known that physical exercise stimulates muscular mitochondrial biogenesis, the increased ADP/ATP ration activates AMPK which proliferate mitochondria, leading to increased resistance to fatigue and enhanced endurance performance. The first studies were made by comparing the mitochondrial mass in different muscle groups with different properties. Despite being less investigated in other tissues; mitochondrial biogenesis occurs in all cells in response to various stimuli and at a various rate in all tissues and it is reasonable to believe that factors influencing biogenesis in one tissue also does so in other tissues. The brain, being highly metabolic active has an intense demand for energy by mitochondrial respiration. Mitochondrial biogenesis is influenced by a variety of triggers influencing the cells aerobic set points in general and

specifically, among others, caloric restriction in a process involving PGC1α leading to increased autophagy and the production of more efficient mitochondria with reduced membrane potential, consuming less ATP and producing less ROS leading to decreased energy expenditure (Onyango et al, 2010). The natural polyphenolic compound resveratrol known for its phytoestrogenic and antioxidant properties has been shown to increase mitochondrial biogenesis by increasing SIRT1 through allosteric interactions. Exercise as aforementioned is well known to stimulate mitochondrial biogenesis in skeletal muscles. Increasing pyruvate levels have been shown to increase mitochondrial biogenesis in myeloblasts. Excess pyruvate results in increased lactate production and oxidation of NADH to NAD^+. The NAD^+-dependent histone interacts with, and regulates, PGC1α (Onyango et al, 2010). The exact mechanism and pathways of mitochondrial biogenesis varies between tissues but involves the coordinated actions of mtDNA and nucleus encoded gene products including NRF-1, NRF2, Tfam and PCG1α. PGC1α often serves as the master regulator. NRF 1 and 2 are PGC1α coactivator factors and their activation coordinates expression of genes encoding mitochondrial proteins. To measure the intensity of mitochondrial biogenesis, the mtDNA number is measured (Klinge, 2008).

8.2.1 Mitochondrial biogenesis and E2

Studies on OVX mice treated with E2 showed an upregulation of NRF-1 in cerebral blood vessels suggesting that E2 may regulate NRF-1 transcription. Recent studies have shown an increase in NRF-1 mRNA expression was increased after E2 treatment in E2 responsive breast cancer cell lines with the conclusion that NRF1 is a primary E2 responsive gene. E2 increases the interaction of both ERα and ERβ with a promoter region of the human NRF1 gene, however ERβ antagonists did not inhibit E2 induced NRF-1 transcription while ERα antagonists did, indicating ERα to mediate the increased NRF1 transcription in the studied breast cancer cell lines. However contradictory results have been presented showing the completely opposite suggesting there may de coregulatory in a ligand and cell dependent manner required for the interaction of ERα and ERβ with the NRF promoter. It may also be tissue variations (Klinge, 2008)

Estogen is able to increase Tfam, necessary for mtDNA replication. Knockdown of NRF-1 inhibited the E2 mediated increase in mtDNA, indicating that mitochondrial biogenesis is mediated by NRF-1 and not directly by E2 (Klinge, 2008).

The E2 mediated increase of mitochondrial biogenesis may be cell specific. Studies on male mouse heart following trauma haemorrhage showed that E2 and the ERβ agonist DNP increased mitochondrial biogenesis, mtDNA transcription and MRC activity. E2 also increased PGC-1α in the trauma haemorrhage- mouse heart model (Onyango et al, 2010).

The fact that the E2 mediated effects on mitochondrial biogenesis may be tissue specific and that no studies are done yet, on hypothalamic mitochondria, makes it hard to draw firm conclusions, however its importance is indicated.

9 Mitochondrial uncoupling and E2

Uncoupling proteins are a family of mitochondrial anion carrier proteins found on the inner membrane of mitochondria in all mammals and plants. The term uncoupling proteins was originally used for UCP 1 which is only found in brown adipose tissue, abundant in hibernating animals and new born and present to in a small amount in adults declining with age and responsible for non-shivering thermogenesis. The UCP1 act as a proton carrier that when activated creates a shunt between complexes of the respiratory complexes and the ATPase. The leak of protons uncouples substrate oxidation from phosphorylation of ADP to ATP leading to an enhancement of cellular respiration and a futile cycle where energy is deliberated as heat. UCP2 has a wider tissue distribution and are present lymphoid tissue, pancreatic islet cells and the central nervous tissue. UPC3 is mainly expressed in skeletal muscles. When discovered, it was hypothesized that UCP 2 and 3 had somewhat similar roles as UCP1 and where involved in non-shivering thermogenesis. The hypothesis was strengthened by results showing a proton pump activity of UCP2 and 3 but such results where only observed when then UCP 2 and 3 where expressed in much higher levels than in normal tissues and its now accepted that they are not involved in the non-shivering thermogenesis but may still contribute to the basal metabolic rate, maybe because a basal leak of protons is associated with the BMR in most tissues (Rousset et al, 2004)

Richard et al (1999) Investigated the distribution of UCP2 in rat and mice brain through *in situ* hybridisation histochemistry methods and found abundant expression of UCP2 mRNA in the ventral septal region, the hypothalamus, the hindbrain (medulla), the ventricular regions and the cerebellum. In hypothalamus an intense hybridization signal was apparent in the suprachiasmatic nucleus, the medial paraventricular region, in the medial parvicellular and magnocellular lateral parts of the paraventricular hypothalamic nucleus and in the arcuate nucleus. The distribution pattern found is highly suggestive for the involvement of UCP2 in neuronal circuits involved in neuroendocrine functions, including energy expenditure, glucose homeostasis and reward behaviour (Richard et al, 1999). The transcription of the UPC2 gene is regulated by several factors such as fatty acids through peroxisomal proliferations activated receptors (PPARs). PPARq agonist enhances UCP2 expression in adipocytes and hippocampus. Posttranslational increase in UCP2 expression has been demonstrated by fatty acids and ROS formed during

oxidative phosphorylation. Furthermore, it has been shown that UCP2 is a negative regulator of mitochondrial hydrogen peroxide. Still unclear how ROS regulates the expression of UCP2 expression a recent theory is that when ROS levels are at tolerable levels UPC2 is conjugated with glutathione, keeping UPC2 in an inactive state. When a slight increase in ROS levels occurs, UPC2 is deconjugated from glutathione and allows an increased proton leak in state 4 respiration. It has also been proposed that an additional role of UCP2 is allowing transport of C-4 metabolites (malate, oxaloacetate and aspartate) and by doing so causing a reduction in substrates for the TCA and thereby attenuate the activity of the electron transport chain, ATP production and ROS production.

UCP2 is expressed in both POMC and NPY/AgRP neurons being an important regulator of neuronal function. With the ablation of UCP2 decreased food intake is observed due to a reduction in NPY/AgRP activation due to elevated ROS levels, due to an increased FA oxidation. The prevention and control of the elevated ROS levels enables the neurons to sustain a high activity level despite the body being in a state of negative energy balance. After intracerebroventricular injection of ROS and ROS scavengers it was demonstrated that ROS increased and ROS scavenger decreased the expression of POMC neuronal activation. It has been suggested that the activation of UPC in obese patients, by preventing an increase in the elevation of ROS in POMC neurons impairs the activity of these neurons during high glucose and leptin levels, causing leptin resistance (Toda & Diano, 2014).

To maintain the tight control of glucose levels in the body the brain has to sense changes in glucose and activate appropriate mechanism for its regulation. Changes in glucose levels are detected in the brain by glucose excited (GE) and glucose inactivated (GI) neurons. Arcuate POMC neurons are one example of GE neurons and NPY/AgRP an example for GI neurons. In case of high level of glucose in POMC neurons, glucose is converted to ATP causing high ATP levels resulting in the closure of Katp channels and depolarization of the neurons. Inhibition of UCP2 has been shown to induce excitation of POMC neurons. This effect was not seen in case of Katp channel deficiency, indicating that UPC2 decreases glucose sensation through the regulation of ATP levels and associated regulation of Katp channels. A similar mechanism of glucose sensing has been observed in MCH-expressing neurons of the lateral hypothalamus. MCH

specific UCP2-KO mice showed increased glucose tolerance and enhanced glucose dependent depolarization of MCH neurons (Toda & Diano, 2014)

Rodriguez-Cuenca (2002) Conducted studies on the sex dependent thermogenesis in young rats and found that female rats had greater oxygen consumption and higher UCP1 content in their brown adipose tissue. These results raise the question if, and how E2 influences the expression/activity of UCPs. On the basis that E2 is known to increase ROS through unknown mechanism and that UCPs are key regulators of mitochondrial energy efficiency and ROS production. Sastre-Serra et al, (2010) investigated the presence of E2 receptors and UCPs in ER positive and ER negative breast cancer cell lines. They found that after E2 treatment of the ER positive cell line, an increased ROS production and a decreased level of uncoupling proteins suggesting that E2 may increase ROS production through an ER-dependent mechanism involving the repression of UCPs (Sastre-Serra et al, 2010).

This is another area in which recent research indicates an E2 influences the mitochondria, however, the primary focus of the recent literature is not on the hypothalamus, but with the known importance of UCP2 in the hypothalamic neurons and the observations that estrogen is able to influence UCPs in other tissues indicates that more research could lead to new and interesting observations.

10 E2 and the mitochondrial ROS levels

Mitochondrial oxidative phosphorylation is together with NADPH oxidases (NOXs) the major cellular source of ROS (Dunn et al, 2015). ROS are formed as a by-product of oxidative phosphorylation, over 90% of oxygen is consumed by mitochondria and generate during normal oxidative phosphorylation 1-4% incompletely reduced oxygen that can react to form ROS. ROS, including the superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2) as well as hydroxyl radicals (OH^-) causes damage to macromolecules such as lipids, protein and DNA (Rettberg et al,2014).

Superoxide is produced by respiratory complex I, II and III and converted to hydrogen peroxide by superoxide dismutase 2. NADPH oxidases transfer electrons from NADPH to oxygen and thereby generating superoxide converted into hydrogen peroxide by superoxide dismutase 3. The hydrogen peroxide generated by the respiratory chain and NADPH oxidase is then converted to reactive OH-radicals (Tian et al, 2015).

Several other enzymatic systems and organelles generates ROS, however to a lesser extent compared to oxidative phosphorylation, among others Xanthine oxidase, and peroxisomes at endoplasmic reticulum (p450) (Okoh et al, 2011). Being the main source of ROS, the mitochondria are also the greatest sufferer of oxidative stress when increased levels of ROS are generated. Many mitochondrial structures are sensitive to oxidative damage such as lipid peroxidation of mitochondrial membranes, and damage to the proteins of the electron transport chain, such damage may result in impairment of mitochondrial function or apoptosis through activation of ASK1/JNK/p38 MAPK pathways (Rettberg et al, 2014; Tian et al, 2015). Low, physiological levels of cellular ROS production are critical for proper induction of neuronal plasticity and as a messenger in cell to cell communication and for cell proliferation (Okoh et al, 2011).

Thus, ROS production has to be well balanced, mainly through a fine- regulated and properly functioning oxidative phosphorylation. Many antioxidant enzymes and processes in the mitochondria and cytoplasm act to balance the redox processes by scavenging radicals and decreasing their production. Such antioxidants are, among others, vitamin C, vitamin E, glutathione peroxidase, and superoxide dismutase.

Present evidences suggest that E2 affects mitochondrial ROS production by both the classical genomic pathway, via nuclear ER receptors and transcription of downstream target genes for the respiratory chain units and through the rapid, non-genomic ways, the latter involving direct interaction with subunits of the respiratory chain, MAPK, calcium (Arnold et al, 2012).

The long term effects of E2 deficiency have been mimicked by OVX in rodents and the result where decreased brain mitochondrial respiration accompanied by decreased oxidative stress. E2 treatment of the aforementioned mice increased certain proteins in the respiratory chain (COX, F0F1-atpase and cytochrome c) accompanied by their increased activity and increased levels of superoxide and consequent oxidative stress. The regulation of mtDNA encoded proteins is not completely elucidated however E2 does increase the transcription of several genes encoding proteins in the respiratory chain through involvement of nuclear ER. E2 also increased the expression of mitochondrial transcription factor (Tfam) and nuclear respiratory factor 1 (NRF-1). NRF 1 and 2 are involved in the increased levels of mitochondrial DNA transcription, an effect mediated by Tfam who is in turn regulated directly by E2. Tfam increases the expression of cytochrome oxidase and ATP synthase genes. Consistent with an increase in cellular respiration through the upregulation of mitochondrial respiration there is also a subsequent increase in superoxide production (Arnold et al, 2012).

In both the NPY and POMC neurons of the hypothalamus, ROS is useful marker in fuel sensing and utilization. Intracellular ROS levels plays a role in in the control of the cellular activity of NPY/AgRP neurons and POMC neuronal thereby regulating food intake and body weight (Gyengesi et al, 2012).

Intriguingly, E2 plays a dual role and does not only induce ROS overproduction but also potentiates the antioxidant defence. E2 enhanced the production of the antioxidant NO by the activation of neuronal nitric oxide synthase in both neuronal and non-neuronal cells (Tian et al, 2015). *In vitro* experiments have shown that E2 were able to protect against DNA damage caused by H2O2 and arachidonic acid. E2 also increased the expression of several antioxidants including superoxidedismutase, peroxiredoxin and glutaredoxin with an associated reduction of ROS. OVX in rodents also caused an increased level of lipidperoxidation that could be prevented by E2 treatment (Rettberg et al,2014).

ROS generation and its consequences, ROS in the hypothalamus and E2 and ROS are relatively well investigated areas in its own, especially in connection to neurodegenerative diseases and cancer (Kim et al, 2015; Okoh et al, 2011; Simpkins & Dykens, 2008), but the combination of these areas need further research.

11 Mitochondrial calcium homeostasis and E2

11.1 Function of neuronal calcium

Calcium (Ca^{2+}) is a universal secondary messenger involved in the regulation of many important cellular functions, including phosphorylation and dephosphorylation of many enzymes controlling the metabolism, cell secretion of different substances, motility processes, exocytosis and apoptosis. In neuron, the most important function of calcium is its role in transmission of the depolarizing signal and its contributions to synaptic activity by the release of neurotransmitters conveying signals across the synaptic cleft to the next neuron. Calcium penetrates the axonal terminal as a result of the opened voltage gated calcium channels; it then promotes fusion of the synaptic vesicles with the presynaptic plasma membrane. Being crucial for, among other, such a fundamental function as the neuronal transmission, the calcium regulation in neurons is controlled by a finely tuned system and neurons possesses delicate calcium signalling pathways.

11.2 Mitochondrial calcium

Calcium enters the neurons through plasma membrane receptors and voltage gated ion channels. The release of calcium from intracellular stores such as mitochondria and endoplasmic reticulum also raises the calcium levels. Calcium levels are regulated within the cells primarily by a constant uptake and release from mitochondria and endoplasmic reticulum (Brini et al, 2014). Mitochondrial calcium uptake is critical for neuronal function, the mitochondria as a major player of the calcium regulation, participate both in the calcium buffering and in the communication with the endoplasmic reticulum that allows them to use localized calcium by endoplasmic reticulum channels allowing them to accumulate calcium in the matrix.

Basal calcium level in neurons is very low. Synaptic activity results in high calcium levels in the cytosol causing calcium influx to the mitochondria (Rettberg et al, 2014). Synaptic activity is an energy demanding process and the increased calcium in the mitochondrial matrix enhances the activity of the citric acid cycle and the ATP production and thus facilitating the removal of calcium from the cytosol by ATP demanding pumps. The main way that calcium enters the mitochondria is via mitochondrial calcium uniporter (MCU) of the inner mitochondrial membrane (Lobatón et al, 2005: Brini et al, 2014). MCU is driven by the negative membrane potential generated by the respiratory chain. The outflow of calcium is through a mitochondrial

sodium/ calcium exchanger and by transient opening of the mitochondrial permeability transporter (Brini et al, 2014).

Oxidative stress in neurons causing, in addition to direct oxidative damage of proteins and lipids, a rise in intracellular calcium and the releases excitatory amino acids such as glutamate. Calcium is known to be involved in cytotoxicity, however the underlying mechanisms are not completely elucidated, there are evidences for a number of toxic environmental chemicals interacts with calcium singling pathways and thereby alter them and ultimately causes apoptosis either by an involvement of calcium mediated expression of ligands binding to and activating death receptors or by having direct toxic effects primarily targeting endoplasmic reticulum and mitochondria. The increase in mitochondrial calcium, enhancing the activity of the respiratory chain will in turn result in increased ROS production (Gyengesi et al, 2012).

11.3 E2 as a modulator of mitochondrial calcium

Estrogens seem to modulate mitochondrial calcium balance direct and indirectly. Mitochondrial overloading with calcium causing malfunction of the mitochondria and ultimately cell death. The roles of E2 as an enhancer of the cellular respiratory activity and concurrent elevation of ROS production, along with evidences that increased ROS production leads to increased levels of mitochondrial calcium through opening of by activating plasma membrane cation channels, suggests a role of E2 in the influx of calcium to mitochondria (Arnold et al, 2012; Brini et al, 2014). Lobatón et al. (2005) showed that several natural phytoestrogens strongly activate the MCU, and that several agonists and antagonists of E2 receptors were able to bind to and activate this channel and thereby enhancing the calcium influx to the mitochondria. The activation was seen immediately and the inhibition reached its maximum after only five minutes, suggesting rapid non-genomic E2 effects.

However, E2 seems to have a dual role in the mitochondrial calcium regulation. E2 is able to increases sodium-dependent calcium efflux, suggesting that E2 have the potential of preventing calcium overload in mitochondria and consequent cellular damage (Lobatón et al, 2005).

12 E2 and mitochondrial membrane potential

Mitochondria are an ultimate requirement for cellular energy production and survival but may also be the initiator of apoptotic cellular death. Mitochondria contains key regulators of caspases, a group of proteases being a major player in many apoptotic processes. The release of cytochrome c from the inner membrane causes assembly of the apoptosome required for the activation of caspases. Decreased membrane potential leads to condensation of the matrix and exposure of cytochrome c to the intermembrane space, facilitating its release and consequent cell death (Gottlieb et al, 2003).

Mitochondria generate ATP by utilizing the proton electrochemical gradient potential, or electrochemical proton motive force, generated by the electron transport chain. The reductive transfer of electrons through the respiratory chain provides energy to drive protons against their concentration gradient across the inner mitochondrial membrane. The accumulated hydrogen ions outside the membranes then flow back into the mitochondria through the F1/F0 ATP synthase (complex 5) producing ATP. The total driving force is a sum of the mitochondrial membrane potential ($\Delta\psi m$) and the mitochondrial pH gradient (Δp). Together these factors are important regulators of mitochondrial energy metabolism, intracellular ion homeostasis and apoptosis. The $\Delta\psi m$ component of Δp regulates ROS production and provides the charge gradient necessary for mitochondrial Ca^{2+} sequestration. Cellular stress, such as nutrient or oxygen deprivation, may cause dysregulation of $\Delta\psi m$ by intracellular ions such as K^+ and Ca^{2+} and consequently change (Δp) and thus ATP production. When the ionic changes are large then the mitochondrial buffer capacity the $\Delta\psi m$ collapses leading to a failure in ATP production, cytochrome c release and apoptosis (Perry et al, 2011).

12.1 Effects of E2 on $\Delta\psi m$ in neurons

Gottlieb et al, (2003) performed an experiment to determine the effects of mitochondrial toxins and E2 in neuronal cultures. First they analysed rhodamine, a mitochondrial specific dye and demonstrated that the toxin, 3NPA caused mitochondrial depolarization. This depolarization was prevented by E2 pre-treatment. They also observed that the calcium concentration required for $\Delta\psi m$ was increased after E2 treatment. This increase could be explained by a partial resistance of all mitochondria to Ca^{2+} or by complete resistance of a group of mitochondria in presence of E2.

Collectively these results indicate that E2 have the ability to prevent Δψm collaps and thus preventing apoptosis (Simpkins & Dykens, 2008).

There are only few publications on this area but it points out a further area in which E2 is able to influence mitochondrial functions.

13 Mitochondrial permeability and E2

Mitochondrial overload by oxidative stress, elevated calcium levels, disputed $\Delta\psi m$, elevated phosphate levels and adenine nucleotide depletions leads to the opening of a non-specific pore in the inner mitochondrial membrane, the mitochondrial permeability transition pore (MPTP). Opening of the MPTP allows free flow of molecules less than 1500 D into the mitochondria, including protons. The result is uncoupling of oxidative phosphorylation, ATP depletion, influx of small weight molecules causing mitochondrial swelling, disruption of the outer membrane, release of cytochrome c and other pro-apoptotic factors and the subsequent cellular death (Halestrap, 2009).

The MPTP is a protein complex that spans both the outer and the inner mitochondrial membrane. The exact protein and lipid composition of the MPTP is not completely elucidated (Friberg & Wieloch, 2002, Baines et al, 2007) It was long believed that the adenine nucleotide translocase (ANT) located in the inner mitochondrial membrane and the protein voltage dependent anion channel (VDAC) located on the outer mitochondrial membrane and cyclophylin-D in the matrix was core components, however Baines et al. (2007) demonstrated that the MPTP was formed in VDAC-null mice. These data indicate that VDAC is not required for the MPTP formation, at least in vitro.

Increased synaptic activity, which elevates calcium levels and causes massive mitochondrial calcium sequestration, causes a transient activation of MPTP in neurons under physiological conditions. Pathophysiological conditions such as cerebral ischemia elevating calcium and ROS levels activates MPTP as well as reperfusion after myocardial infarction and neuronal exotoxicity and thus mediate cell damage (Baines et al, 2007).

13.1 E2 and the mitochondrial permeability transition pore

The influence of E2 on the mitochondrial permeability transition pore has been widely researched in association with its cardio protective roles following myocardial infarction and reperfusion injuries. Morkuniene et al. (2010) demonstrated that treatment of heart muscle with E2 increased the resistance of isolated mitochondria to calcium induced MPTP opening measured as calcium retention capacity. The effects of E2 were abolished by the cGMP-dependent protein kinase inhibitor KT823 indicating that the effects are mediated by cGMP-dependent protein kinase. The effects of E2 on MPTP is the same as for Cyclosporin A which is known to targetcyclophilin D,

this may imply that E2 triggered cascade affects cyclophillin D. Most studies have focused on the heart but it has been demonstrated that activated cGMP dependent protein kinase can block the MPTP in isolated brain and liver mitochondria (Morkuniene et al, 2010).

14 E2 as an antiapoptotic agent

Apoptosis can be initiated through either intrinsic or extrinsic pathway leading to caspase activation. Mitochondria play key roles in activating apoptosis in mammalian cells. E2 is able to prevent apoptosis by regulation of previously described mitochondrial processes, that when not properly tuned, causes cellular stress and activate the intrinsic pathway of apoptosis.

The intrinsic mitochondria-dependent pathway is regulated by regulatory protein Bcl-2 family members governing the mitochondrial outer membrane permeabilization responsible for cytochrome c release. The members of the Bcl-2 family either promote or inhibits apoptosis depending on their interaction with prosurvival family members like Bcl-2, Bcl-xL and McI-1 or pro apoptotic members like Bax, Bak, Bok, Bid, Puma and Bad (Klinge, 2008).

Schacter et al, (2014) studied E2´s ability to increase the expression of the prosurvival protein McI-1 in ERα+ breast cancer cell lines, They found McI-1 mRNA to be two-fold increased after E2 treatment. Treatment with the antiestrogens tamoxifen and Fulvestrant in combination with E2 failed to increase MCI-1 mRNA and knockdown of ERα using serum starved MCF-7 cells transfected with small interfering (si)RNA against ERα. Knockdown of ERα blocked E2 induced Mc1-1. Overall this suggest that ERα plays an important role in regulating the Mc1-1 mRNA expression. Several studies have indicated that E2 may upregulate Bcl-2. E2 overexpression of Bcl-2 seems to be regulated via ERα as both Tamoxifen and Fulvestant could decrease Bcl-2 expression. Another study with cultured hippocampal neurons showed that E2 increases Bcl-xL expression through a mechanism involving an ERE site within the Bcl-XL gene (Schacter et al, 2014).

Beyond doubt, E2 plays an important role as a modulator of cell survival and apoptosis. Its ability to influence critical mitochondrial processes such as bioenergetics, calcium regulation, ROS production, permeability and membrane potential together with the ability to upregulate certain prosurvival proteins are all examples of its involvement in cell survival.

From literature, it is evident that the role of mitochondrial dysfunction is important in many neurodegenerative diseases, such as Parkinson disease and Alzheimer’s disease and that E2

treatment could improve mitochondrial function and could be beneficial for patients suffering from these diseases, making it a hot research area (Simpkins & Dykens, 2008; Engler-Chiurazzi, 2016).

However, the importance in hypothalamic neurons needs to be further investigated.

15 Conclusions and discussion

The aim of this literature review was to understand the multifaceted role of E2 and its effects and possible effects on mitochondrial contribution to central metabolic pathways and on specific mitochondrial functions from the perspective of hypothalamic point of view with particular attention paid to E2's role on mitochondria in hypothalamic neurons.

The topic is scarcely researched; studies devoted on E2's effect on hypothalamic mitochondria are to my knowledge, up to today, non-existing. However, E2's effects on mitochondria, mitochondrial function in hypothalamus and E2 in hypothalamus are investigated separately and by extrapolating the results from the different areas possible effects of E2 on hypothalamic mitochondria can be concluded. To validate the possible effects more specific studies on hypothalamic mitochondria are needed.

There is no doubt that E2 have direct and indirect effects mitochondria of virtually all cells in the body through nuclear receptors, extranuclear E2 receptors and via non-genomic interactions with other structures/proteins and that E2 receptors are abundant in hypothalamus and E2 readily permeates the blood brain barrier and is locally synthetises in the brain, thus we always have to consider a concerted E2 action on the mitochondrial performance and it is likely that mechanisms studied in other tissues, and particularity in other areas of the brain, are valid for hypothalamus as well.

E2 influences cellular energy metabolism by enhancing GLUT expression and upregulate many key enzymes for the glycolysis and the respiratory chain which makes it reasonable to say that E2 is able to increase the cellular energy production when needed and sustain energy supply of cells when energy deprived thus acting as neuroprotective. Hypothalamic synapses are highly active and thus in need of great amount of energy.

E2 has also been shown to influence the mitochondrial biogenesis, fission and fusion, possesses vital to ensure the higher density in areas with high energy need and involved in apoptosis. E2 deficiency has been shown to increase mitochondrial fission and E2 treatment does upregulate many fusion and fission proteins hence E2 is able to regulate the number and conformation of mitochondria.

E2 does increase the levels of ROS by increasing the cellular respiration through upregulation of certain proteins involved in cellular respiration but seems to play a dual role by subsequently potentiating the antioxidant defence. In hypothalamic neurons, ROS levels control the cellular activity of NPY/AgRP and POMC neurons and thereby regulating food intake and body weight. E2 thus seems to increase ROS levels, being beneficial as regulator of certain processes and subsequently protect the cells from damage caused by excessive ROS.

Basal calcium levels in neurons is low. Synaptic activity results in high calcium level causing an influx of calcium into mitochondria. E2 is able to increase the influx of calcium to mitochondria thus regulating the cellular calcium balance. Mitochondrial calcium increases the efficiency of cellular respiration and energy production but in excessive amounts, calcium damaged the mitochondria and may lead to dysfunction and ultimately apoptosis. E2 seems to play a dual role and also regulated the outflow of calcium from the mitochondrial thus being important in the fine tuning of cellular and mitochondrial calcium balance.

Much attention has been given to investigate the potential antiapoptotic effects of E2 and it has been shown that E2 is able to prevent collapse of $\Delta\psi m$ thus acting to prevent cellular damage and apoptosis. It has also been shown to increase the resistance of mitochondria to calcium induced opening of the MPTP mostly investigated in cardiac muscle cells because of E2's potential indicator as a cardio protective drugs following myocardial damage and reperfusion injury. One study did however indicate that E2 was able to block the MPTP in isolated brain mitochondria. Results from studies on breast cancer cell lines indicate that E2 may be antiapoptotic by upregulating certain antiapoptotic proteins from the Bcl-2 family, primarily by mediated by ERα, however one study showed similar results on cultured hippocampal neurons. The above does indicate that E2 is able to influence apoptosis through several pathways. However, results from hypothalamic neurons are missing and even though it seems likely that E2 could act antiapoptotic in the hypothalamus there is not enough evidences to conclude such mechanisms.

The concluding thoughts from this review is that E2 is able to influence the mitochondria in the hypothalamus in many different ways and by regulating the morphology and function, ensure proper energy supply and to protect the cells from insult by fine tuning of many mitochondrial processes, regulation of the antioxidant system and by preventing apoptosis. Thus being neurotrophic and neuroprotective. However, the mechanisms are still partly not revealed and

much more research aimed at the specific hypothalamic mitochondrial population is required to truly understand the multifaceted role of E2 on mitochondria in the hypothalamic neurons.

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17 Acknowledgements

First of all, I would like to express my gratitude to my supervisors Dávid Sándor Kiss, PhD & Isván Tóth, DVM, PhD for their useful comments, remarks and encouragement through the process of my thesis work. I would also like to thank Mária Ashaber, PhD who was one of my supervisors and of great help in the beginning of the process, before she moved to the US for new adventures- I wish her the best of luck.

In addition I would like to thank László V. Frenyo, DVM, CSc. Head of the department of physiology and biochemistry for his trust and kindness to let me write my thesis with the department of physiology and biochemistry.

I would also like to thank all the staff at the department for being friendly, helpful and letting me take part in some of the laboratory work at the department to get an introduction into the research work taking place at the department.

Last but not least, I would like to thank my loved ones for their support and help to put pieces together.

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