Protection of neurons – glutathione as a key mediator for survival with potential implication for use of estrogens as neuroprotectors

Thesis for the degree of *Philosophiae Doctor*

by

Irene Beate Sørvik

Department of Pharmaceutical Biosciences

School of Pharmacy

Faculty of Mathematics and Natural Sciences

University of Oslo

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Table of contents

List of papers

Paper I

Rakkestad K.E[1]; **Sørvik I.B[1];** Øverby G.R; Debernard K.A.B; Mathisen G.H; Paulsen R.E (2014). 17α-Estradiol down-regulates glutathione synthesis in serum deprived PC-12 cells. *Free radical research*. ISSN 1071-5762. *48* (10), s 1170- 1178.

[1]These authors contributed equally to this work.

Paper II

Solum E.J; Cheng J-J; **Sørvik I.B**; Paulsen R.E; Vik A; Hansen T.V (2014). Synthesis and biological evaluations of new analogs of 2-methoxyestradiol: Inhibitors of tubulin and angiogenesis. *European Journal of Medicinal Chemistry*. ISSN 0223-5234. *85*, s 391- 398.

Paper III

Sørvik I.B; Solum, E.J; Hansen T.V; Paulsen R.E (2017). Novel estrogen analogs with improved neuroprotective properties in serum-deprived PC12 cells. Manuscript.

Paper IV

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Summary of thesis

Oxidative stress, mitochondrial dysfunction, and glutamate excitotoxicity are common insults shared by many neurodegenerative diseases. Estrogen (17β-estradiol) has neuroprotective properties and exerts various actions on many organs and physiological systems. The neuroprotective properties of estrogen were discovered in the 1990s when estrogen replacement therapy in menopausal women was associated with decreased risk of Alzheimer's disease. Its stereoisomer 17α-estradiol with weak estrogen activity is equally neuroprotective as 17β-estradiol. However, when the benefits of estrogen replacement treatment became controversial due to side effects of estrogen treatment, many estrogen analogs with low estrogen activity have been developed with the motivation for clinical application.

The aim of this thesis was to examine how 17α-estradiol and different estradiol analogs may have actions as neuroprotectors due to possible interference with glutathione, the main endogenous antioxidant in the cell. Serum-deprived PC12 cells and glutamate-induced toxicity in chicken cerebellar neurons (CGNs) were used as models. When 17α-estradiol was used at an antioxidant concentration (10 µM), the steroid enhances deprivation-induced cell death due to down-regulation of the promoter activity of the catalytic subunit of the rate-limiting enzyme in glutathione synthesis, glutamate cysteine ligase (GCLC), and reduction in glutathione levels. In contrast, low concentration (1 nM) protects. The down-regulated glutathione synthesis was due to decreased levels of c-Fos, which is part of the AP-1 transcription factor complex that binds in the GCL promoter, and increased level of activated p38 mitogen activated kinase (MAPK). By modifying 2-methoxyestradiol (2-ME), the estradiol steroid gained improved properties with regards to glutathione regulation at 10 µM. The G protein-coupled estrogen receptor (GPER) antagonist G36 was also protective against deprivation-induced cell death. In the CGNs, 10 µM protected against glutamate-induced cell death while 1 nM did not when administrated together with glutamate. When glutamate was given after a pretreatment period of 24 h, 1 nM protected, but 10 µM failed to protect. The difference in protection was linked to a lower calcium response in the protective treatments. Increased protein level of *N*-methyl-ᴅ-aspartate (NMDA) receptor subunit NR2B, as well as decreased glutathione levels were seen in neurons pretreated with 10 µM. Our results reveal that low concentration of 17α-estradiol has potential as neuroprotector, and provide information about the importance of glutathione as a mediator for neuronal survival.

Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methylisoxale-4-propionate
AP-1	Activator protein-1
ARE	Antioxidant response element
CGNs	Cerebellar granule neurons
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinases
GCL	Glutamate cysteine ligase
GluR	Glutamate receptors
GPER	G protein-coupled estrogen receptor
GS	GSH synthetase
GST	Glutathione-S-transferases
HD	Huntington's disease
HRT	Hormone replacement therapy
MAPK	Mitogen-activated protein kinases
2-ME	2-Methoxyestradiol
MS	Multiple sclerosis
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa B
NMDA	*N*-methyl-ᴅ-aspartate
NOS	Nitric oxide synthase
Nrf2	Nuclear erythroid 2-related factor 2
PD	Parkinson's disease
PC12	Adrenal pheochromocytoma, rat (cell line)
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TNF	Tumor necrosis factor

1. Introduction

1.1 Neuroprotection

There is a desirable aim to slow disease progression for many disorders in the central nervous system (CNS) and to prevent irreversible loss of neurons (Seidl and Potashkin 2011). In order for a neuronal cell to function properly, all of its structures and compartments (e.g. soma, dendrite and axon) must be intact. Hence, neuroprotection may be defined as

"*The relative preservation of neuronal structure/and or function*" (Casson et al. 2012).

This definition can only be understood in light of the actual neuronal threat or insult (Casson et al. 2012). In the brain, extensive production of reactive oxygen species (ROS) is associated with development and progression of neurologic disorders. Many neuronal degenerative diseases like stroke, Alzheimer's disease (AD), multiple sclerosis (MS), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and loss of trophic support share common mechanisms behind neurodegeneration, including oxidative stress, thiol redox imbalance, mitochondrial dysfunction, and glutamate excitotoxicity (Lipton and Rosenberg 1994, Snider 1994, Halliwell 2006, Lau and Tymianski 2010, McBean et al. 2015). These insults often lead to neurodegeneration and cell death. Therefore, an important therapeutic goal in neuroprotection is to target excessive glutamate signaling and mechanisms to reduce oxidative stress.

The focus of this work has been to study neuroprotective effects of 17α-estradiol and estradiol analogs in serum-deprived rat pheochromocytoma (PC12) cells and glutamate treated chicken cerebellar granule neurons cells (CGN) with emphasis on interference with glutathione, the main endogenous antioxidant, and ROS production in PC12 cells in addition to glutamate-induced calcium response in CGNs.

1.2 Oxidative stress

A free radical can be defined as "any species capable of independent existence that contains one or more unpaired electrons" (Halliwell 2006). ROS includes oxygen radicals and certain non-radicals that are oxidizing molecules and/or are converted into radicals, while nitrogen species (RNS) include nitrogen radicals and non-radicals (Halliwell 1996). Living cells generate small amounts of ROS as a byproduct of oxidative metabolism, and the cells continuously repair the damage they cause. Oxidative stress and oxidative damage of molecules occur in situations where the antioxidant defense is not able to cope with the production of ROS/RNS. Oxidative stress has for a long time been defined as an imbalance of pro-oxidants and antioxidants, but a newer proposed definition is "a disruption of redox signaling and control" (Jones 2006). Increases in lipid peroxidation end-products, DNA (and often RNA) base oxidation products, and oxidative protein damage are insults defined as oxidative damage (Halliwell 2001, Halliwell 2002, Moreira et al. 2005, Sultana et al. 2006).

Oxygen is the primary source for free radicals and non-radicals. If a single electron is supplied to O_2, superoxide radical anion $O_2^{\bullet -}$ is formed. $O_2^{\bullet -}$ reacts quickly with nitric oxide ($NO^{\bullet}$) to form a non-radical product, peroxynitrite ($ONOO^-$) that can cause lipid peroxidation, protein oxidation, protein nitration and DNA damage (Radi et al. 1991b, Radi et al. 1991a). In addition, $O_2^{\bullet -}$ inactivates enzymes involved in amino acid metabolism and energy production (Imlay 2003, Liang and Patel 2004). Peroxynitrite can rapidly protonate to the reactive non-radical peroxynitrous acid (ONOOH) at physiological pH. Peroxynitrous acid can also cause damage by oxidizing and nitrating proteins, lipids and DNA (Alvarez and Radi 2003), in addition to undergo homolytic fission to the reactive hydroxyl radical (Halliwell 2006).

$$ONOOH \longrightarrow NO_2^{\bullet} + OH^{\bullet}$$

Oxygen can also be converted to hydrogen peroxide (H_2O_2) through two-electron reduction, and to water through four-electron reduction (Halliwell 2006).

$$O_2, \xrightarrow[\text{reduction (plus } 2H^+)]{\text{two-electron}} H_2O_2 \qquad O_2, \xrightarrow[\text{reduction (plus } 4H^+)]{\text{four-electron}} 2H_2O$$

Hydrogen peroxide can be precursor for the hydroxyl radical ($OH^{\bullet}$) through the Fenton reaction *in vivo*:

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + {}^{-}OH$ (Halliwell 1996)

The toxicity of the different ROS/RNS relates to their selectivity. Superoxide and hydrogen peroxide are highly selective in their reactions with biological molecules, harming only a few molecules in their proximity, while the hydroxyl radical is non-selective and causes damage by attacking everything around it (Halliwell 2006).

However, the oxygen-derived molecules are not always harmful as they have a physiological role in the developing brain. ROS production and ROS signaling during development are important for redox-sensitive molecules such as transcription factors, signaling proteins and cytoskeletal components, and contributes to processes like neurogenesis and differentiation, neurite outgrowth, and neuronal plasticity (reviewed in (Borquez et al. 2016)).

1.3 Defending the brain

Protection of neurons and cells in general is largely dependent on the presence of an endogenous antioxidant system. In order for cellular proteins to function there has to be a stable intracellular redox environment and it is not a coincidence that the evolution of antioxidant defense is intimately connected with the evolution of aerobic respiration (Halliwell 1999). The brain has a high metabolic demand and cells with high oxidative metabolism like the neurons are especially dependent on a functional antioxidant system. Balance between the ROS level and the production and activity of antioxidant defense is important for the integrity of the environment in cells.

One important defense mechanism is to keep the oxygen in an optimal level. Therefore, all parts of the nervous system in animals contain superoxide dismutases (SODs), enzymes that remove superoxide by catalyzing its dismutation to hydrogen peroxide and oxygen (Liochev and Fridovich 2005).

$O_2^{\bullet -} + 2H^{+} \longrightarrow H_2O_2 + O_2$

The active site of SODs in the mitochondrial matrix contains manganese (MnSOD) and SODs in the intermembrane space and in the rest of the cell contain copper and zinc (CuZnSOD) (Liochev and Fridovich 2005). Enzymes important for removing hydrogen peroxide produced by SODs in the brain are glutathione peroxidases (Brigelius-Flohe 1999) and peroxiredoxines

(Rhee et al. 2005). Peroxiredoxines contain an active site cysteine that is sensitive to oxidation by H_2O_2 (Park et al. 2016). Glutathione peroxidases are selenium-containing enzymes that reduce H_2O_2 through oxidation of glutathione (Brigelius-Flohe 1999).

$$H_2O_2 + 2\ GSH \longrightarrow GSSG + 2\ H_2O$$

Hydrogen peroxide can also be reduced by catalase, but this enzyme is not present in the brain mitochondria where superoxide is generated (Halliwell 2006).

1.3.1 Glutathione

The major intracellular antioxidant and reducing agent in the cell is the tripeptide glutathione, γ-L-glutamyl-L-cysteinylglycine. It is the most abundant thiol group in the mammalian cell with a high concentration in intracellular versus extracellular compartments (millimolar vs micromolar) (Meister and Anderson 1983). In the brain, forebrain and cortex seem to have the highest glutathione content, followed by cerebellum, hippocampus, striatum, and substantia nigra (Kang et al. 1999, Calabrese et al. 2002). Because of the high oxidative metabolism in the brain from mitochondrial respiration, the antioxidant function of glutathione is especially important for the brain (Schmidt and Dringen 2012). It is emphasized that the glutathione system is related to events critical for normal embryonic development and differentiation as it acts a meditator of numerous processes (Hansen and Harris 2015). Further, evidences link progression of neurologic disorders such as PD and AD, schizophrenia and bipolar disorders, in addition to various human diseases to a compromised glutathione metabolism (Schmidt and Dringen 2012).

Glutathione exists in two forms, as thiol-reduced (GSH) and disulfide-oxidized (GSSG), and the GSSG content is normally less than 1% of GSH (Akerboom et al. 1982). However, a shift in this ratio occurs under oxidative conditions due to oxidation of GSH to GSSG. Since glutathione is the most abundant non-protein thiol in mammalian cells it protects essential thiol groups in proteins against oxidation by being substrate for thiol-disulfide exchange by thiol-transferase in a reversible reaction (Lu 2009):

$$\text{Protein-SSG} + GSH \longrightarrow \text{Protein-SH} + GSSG$$

Thiol redox imbalance is regarded as a significant contributor to neurodegenerative disease (reviewed in (McBean et al. 2015)). As an antioxidant glutathione serves as an electron donor and reacts nonenzymatically with both free radicals and non-radicals such as hydroxyl radical

and peroxynitrite (Griffith 1999). In addition, glutathione has many roles in cellular functions such as storage and transport of cysteine, protein synthesis, DNA synthesis and repair, cell proliferation, and redox signaling (Meister and Anderson 1983, Wu et al. 2004, Townsend 2007) or via activities of detoxification enzymes like glutathione peroxidases. It is also involved in phase-II-metabolism of xenobiotics via glutathione-S-transferases (GST)-mediated glutathione conjugation reactions (Hayes and Pulford 1995).

The difference in concentration range reflects the different functions of glutathione intracellularly versus extracellularly (Table 1).

Table 1 Putative functions of glutathione in brain

Intracellular functions (mM concentration range)	Extracellular functions (μM concentration range)
Radical scavenger	Substrate of enzymes (γGT)
Electron donor for non-enzymatic reduction	Radical scavenger
Substrate of enzymes (Glutathione peroxidases, GST, isomerases)	Modulator of glutamate receptors
Leucotriene metabolism	Neurohormone
Detoxification of xenobiotics	Transport form of cysteine
Redox buffer	
Covalent modulator of protein functions via S-glutathionylation	
Storage form of cysteine	

Table from (Schmidt and Dringen 2012)

Mitochondrial glutathione is particularly important in defending against both physiologically and pathologically generated oxidative stress due to the absence of catalase in mitochondria (Circu and Aw 2008). Glutathione homeostasis in mammalian cells is regulated by synthesis, uptake from exogenous sources across plasma membranes, utilization, export from the cell and regeneration of GSH from GSSG by glutathione reductase in a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reaction (Griffith 1999, Circu and Aw 2008). Under normal oxidative conditions in the cell, the ratio between GSH and GSSG is maintained by the levels of NADPH and the activity of glutathione reductase. In neurons, NADPH is generated through oxidation of glucose from the pentose phosphate pathway (Fernandez-Fernandez et al. 2012). During increased oxidative stress and/or if NADPH becomes limiting in the regeneration reaction, GSSG may accumulate which will cause a shift in the GSH/GSSG ratio and secretion of GSSG from the cell (Griffith 1999). In the brain, members of ATP-driven multidrug proteins (Mrps) export glutathione and its conjugates

(Schmidt and Dringen 2012). Exported GSSG is degraded extracellularly resulting in loss of GSSG and total glutathione levels intracellularly. The biosynthetic capacity and cellular requirements for synthesis are therefore important in maintaining glutathione homeostasis during oxidative stress (Griffith 1999). More details about glutathione redox cycle are described in Figure 1.

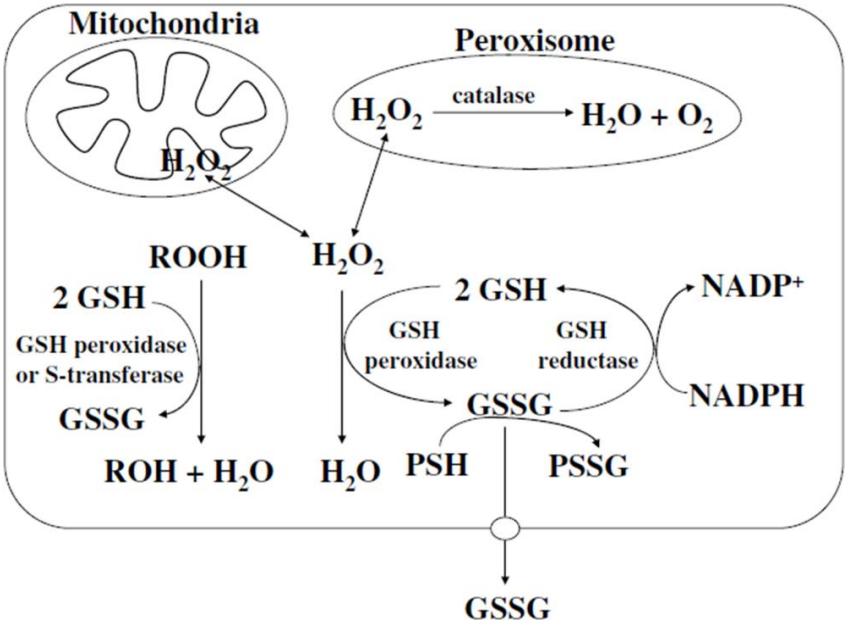

Figure 1 *Antioxidant functions of glutathione. Hydrogen peroxide from aerobic metabolism can be metabolized by glutathione (GSH) peroxidase in the cytosol and the mitochondria, and by catalase in the peroxisome. GSSG is reduced back to GSH by glutathione reductase at the expense of NADPH. Organic peroxides (ROOH) can be reduced either by GST or glutathione peroxidase. Accumulation of GSSG can occur under severe oxidative stress due to decreased capacity to reduce GSSG to GSH. To avoid shift in the equilibrium, GSSG is transported out of the cell or it can react with a protein sulfhydryl (PSH) to form a mixed disulfide (PSSG). Figure and legend from (Lu 2009).*

1.3.2 Glutathione synthesis

The importance of a functional glutathione metabolism is seen in relatively rare cases where inborn errors in glutathione synthesis results in such neurological symptoms as mental retardation and motor functional disturbances (Schmidt and Dringen 2012). Glutathione is synthetized in two ATP-dependent steps in the cytosol. The first step is regulated by the enzyme glutamate cysteine ligase (GLC) composed of a catalytic (GCLC, ~73 kDa) and a modifier (GCLM, ~31 kDa) subunit and the second step by glutathione synthetase (GS)

(Franklin et al. 2009). GCL catalyzes the formation of the dipeptide γGluCys between glutamate and cysteine while GS adds glycine to the dipeptide. GCL is the rate limiting enzyme in glutathione synthesis and is strongly regulated by glutathione feedback inhibition (Richman and Meister 1975). The catalytic subunit GCLC has synthesis activity and GCLM modulates the activity of GCLC. GCLM decreases the K_m for ATP and glutamate, increases the K_i for feedback inhibition by glutathione and K_{cat} for γGluCys (Chen et al. 2005). GCLC is important in dictating the GCL activity, but GCLM is limiting for the GCL holoenzyme formation and enhanced expression of GCLM will also enhance the GCL activity (Franklin et al. 2009). Since GCL is considered rate-limiting, GS has been given less attention. However, GS is also important in determining the overall glutathione synthesis since increased GS expression is reported to further enhance the capacity to synthesize glutathione (Lu 2009).

Glutathione synthesis relies on the availability of its three amino acids and cysteine is often limiting. Also, since glutamate and glycine are neurotransmitters the extracellular availability in the brain of these are kept low (Schmidt and Dringen 2012). Storage of cysteine in glutathione is an important function since cysteine is unstable and auto-oxidizes to cystine extracellularly, a process which also causes formation of free radicals (Lu 2009). For glutathione synthesis, mature neurons depend on extracellular cysteine provided from neighboring cells while astrocytes are able to utilize and convert cystine, different amino acids and peptides taken up by respective transporters as source (Schmidt and Dringen 2012, Dringen et al. 2015). Uptake of cystine occurs via the plasma-membrane x_c^- cysteine-glutamate exchanger in a 1:1 ratio, and this exchanger has therefore an important role in regulating extracellular glutamate (McBean et al. 2015).

Metabolic cooperation between astrocytes and neurons is important for glutathione homeostasis (Figure 2). Astrocytes export glutathione and make it substrate for γ-glutamyl transpeptidase (γGT) that catalyzes the transpeptidation and hydrolysis of the γ-glutamyl group of glutathione producing CysGly (Ikeda and Taniguchi 2005). CysGly can be taken up by the peptide transporter PepT2 which is expressed in astrocytes, neurons and throughout the brain (Shen et al. 2004). However, cultured neurons do not take up CysGly and it is suggested that aminopeptidase N (ApN) cleaves CysGly to cysteine and glycine which can be taken up by sodium dependent transporters into the neurons (Schmidt and Dringen 2012). Further, it is strongly suggested that γ-glutamyl transpeptidase-dependent generation of cysteine from glutathione occurs *in vivo* (Schmidt and Dringen 2012), thus making astrocytes important for glutathione homeostasis in the neurons.

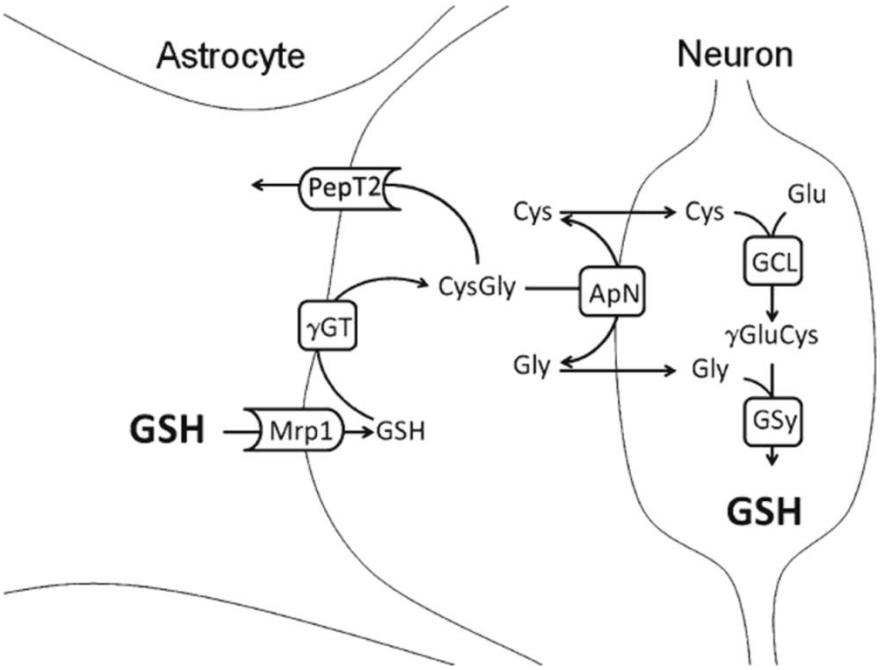

Figure 2 *Metabolic interactions between astrocytes and neurons in the glutathione metabolism. Astrocytes provide the extracellular cysteine required for neuronal glutathione synthesis through export of glutathione via Mrp1 or other transporters. Extracellular glutathione is used as substrate of the astrocytic ectoenzyme γ-glutamyl transpeptidase (γGT) that produces the dipeptide CysGly. This peptide can be hydrolyzed by neuronal aminopeptidase N (ApN) and the generated amino acids cysteine and glycine are taken up into neurons. CysGly can also be taken up into astrocytes by the peptide transporter PepT2. Figure and legend from (Schmidt and Dringen 2012).*

1.3.3 Regulation of glutathione

Transcriptional regulation of GCL is important for glutathione synthesis. The human promoter of GCLC contains several response elements like activator protein-1 (AP-1), activator protein-2 (AP-2), antioxidant response element (ARE), nuclear factor kappa B (NFκB), and metal response (Lu 2009). The GCLM promoter shares many of the same elements. They are all regulated by trans-acting regulatory transcription factors, including nuclear erythroid 2-related factor 2 (Nrf2), Nrf1, AP-1, NFκB, Maf family proteins, JunD, Fra, cAMP response element-binding protein (CREB), and others (Lu 2009). The ARE element is identified as important for induction of the GCLC gene by transcription factors Nrf1 and Nrf2 during oxidative stress (Lu 2009). Nrf1 is localized to the membrane of the endoplasmic reticulum while Nrf2 is kept in the cytosol by Keap1 under normal stress conditions regulated by ubiquitination and proteasomal degradation (Cullinan et al. 2004, Wang and Chan 2006).

However, during endoplasmic reticulum stress Nrf1 moves to the nucleus and activates gene expression (Wang and Chan 2006), while Nrf2 undergoes a translocation from Keap1 to the nucleus when the cell senses oxidative stress and binds to ARE, resulting in increased levels of GCLC, GCLM and other target genes (Kobayashi and Yamamoto 2006). The importance of Nrf1 and Nrf2 is shown through Nrf1 and/or Nrf2 knockouts having lower GCLC expression, lower GSH levels and reduced capacity towards oxidative stress (Lu 2009). Also, in animals deficient in Nrf2, acute oxidative stress is increased during stroke and traumatic brain injury and insufficient Nrf2 activation has been linked to diseases such as PD, AD and ALS (Sandberg et al. 2014).

AP-1 which contains members of c-Jun and c-Fos proto-oncogene families, and others binds to AP-1 sites (Angel and Karin 1991). In promoters lacking ARE increased activity of the rat GCLC promoter by *tert*-butyl hydroquinone was observed and AP-1 activity was identified to be essential for this induction (Yang et al. 2002). It was later shown that Nrf1 and Nrf2 regulate the GCLC promoter by modulating the expression of AP-1 and NFκB family members in the absence of ARE, suggesting cross-talks between the different families of transcription factors (Yang et al. 2005).

Induction of glutathione synthesis can also be regulated through mitogen-activated protein kinases (MAPKs) (Kong et al. 2001). Signaling through MAPKs is regulated by phosphorylation and dephosphorylation on serine and/or threonine residues (Genestra 2007). They respond to oxidative stress (Genestra 2007), but are also important in the regulation of a variety of cellular processes such as cell differentiation, proliferation and cell death (Blenis 1993). There are four members of MAPKs, namely extracellular signal-regulated kinases 1/2, (ERK1/2), c-Jun N-terminal kinase/stress activated protein kinase (JNK), p-38 and the big mitogen-activated protein kinase 1 (Genestra 2007). MAPKs have been shown to regulate glutathione synthesis and glutathione levels through the Nrf2-ARE axis (Kachadourian et al. 2011, Correa et al. 2012), but also independently of Nrf2 (Huseby et al. 2016). In astrocyte-rich cultures, it is shown that activated histone deacetylases induced by prolonged inflammation down-regulates Nrf2-activation via the kinases p38 and glycogen synthase kinase 3-beta, while the kinases ERK1/2 and c-Jun N-terminal kinase up-regulate the Nrf2-system (Correa et al. 2012, Sandberg et al. 2014). The authors hypothesize that this type of dysregulation of Nrf2 system occurs in the brain. Therefore, the MAPKs may have both protective and non-protective effects in regard to glutathione metabolism.

The rate of glutathione synthesis is also determined by distinct transcriptional and post-translational modifications of both GCLC and/or GCLM subunits (Franklin et al. 2009). In addition to activation or suppression of gene transcription, enhanced protein translation can be promoted by increased mRNA stability. After translation reversible holoenzyme formation of the two subunits of GCL is promoted by an oxidative environment and inhibited by a reducing environment (Franklin et al. 2009). It is suggested that cysteine-553 in GCLC is involved in the heterodimer formation between GCLC and GCLM (Tu and Anders 1998) and that covalent modification of cysteine-553 also increases GCLC's enzymatic activity (Backos et al. 2011). Increased GCL activity has also been shown to be caused by decreased glutathione levels without any detectable changes in subunit protein levels (Ochi 1995, Ochi 1996). Glutathione synthesis is also regulated through negative feedback regulation by binding of glutathione to the glutamate site of GCLC (Richman and Meister 1975). Autophosphorylation and kinase-mediated phosphorylation of GCLC are other ways to rapidly regulate GCL activity (Franklin et al. 2009). Also, other known post-translational modifications are the cleavage of GCLC to a stable 60 kDa fragment in caspase-dependent apoptosis in addition to myristoylation of the 13 kDa C-terminal fragment of caspase cleaved GCLC and/or altered subcellular localization (reviewed (Franklin et al. 2009)).

1.4 Cell death

Cell death occurs naturally during normal development in multicellular organisms. During embryogenesis, there is a balance between removal of neurons and surviving cells to ensure that pre- and postsynaptic connections are formed properly (Yuan et al. 2003). Upon neuronal maturation, immature neurons express transmembrane factors in addition to signaling molecules that regulate the cell survival/death decision (Pfisterer and Khodosevich 2017). However, excess neuronal death also occurs in pathological and neurotoxic conditions due to genetic or accidental factors in various neurodegenerative disorders (Lipton and Rosenberg 1994, Yuan et al. 2003).

Traditionally, cell death has been divided into apoptosis and necrosis based on morphological criteria. Apoptosis was first introduced by (Kerr et al. 1972) and is characterized by rounding-up of the cell, reduction of cellular volume, chromatin condensation, plasma membrane blebbing, DNA fragmentation, and formation of membrane bound apoptotic bodies which are engulfed by neighboring cells (Kroemer et al. 2009). Apoptosis is critical in the turnover of cells in tissues as well as during normal development and senescence (Franco and Cidlowski

2009). Necrosis is characterized by swelling of organelles, plasma membrane rupture and loss of intracellular contents, where triggering factors result in mitochondrial overload and dysfunction (Kroemer et al. 2009). The loss of membrane integrity and the release of noxious cellular constituents lead to inflammation in the surrounding tissue (Orrenius et al. 2003). However, the picture is more complex as a dead cell may have both characteristics of apoptosis and necrosis in addition to that the same causative factor can trigger either apoptotic or necrotic cell death (Orrenius et al. 2003). During the last few years, it has become clear that apparently similar death morphotypes often hide a great degree of functional, biochemical and immunological heterogeneity (Galluzzi et al. 2012). Therefore, there has been a switch from morphological to molecular definitions of cell death modalities, and the Nomenclature Committee on Cell Death proposed in 2012 a functional classification of cell death that includes extrinsic apoptosis, caspase-dependent intrinsic apoptosis, regulated necrosis (necroptosis), autophagic cell death, and mitotic catastrophe (Galluzzi et al. 2012).

1.4.1 Apoptosis

Both intrinsic and extrinsic apoptosis rely on the activation of the cysteine-dependent aspartate specific proteases (caspases) which exist as zymogens (procaspases) that undergo dimerization or cleavage upon activation (Riedl and Salvesen 2007). The extrinsic apoptosis is also referred to as the death receptor mediated pathway. It is induced by extracellular stress signals such as tumor necrosis factor α (TNFα), FAS/CD95 or TNF-related apoptosis inducing ligand (TRAIL) that bind to various death receptors on the plasma membrane (i.e., FAS/CD95, TNFα receptor 1 (TNFR1) and TRAIL receptor (TRAILR)1-2, respectively (reviewed by Galluzzi et al. 2012). The binding of the ligand promotes the formation of the death inducing signaling complex (DISC) which recruits caspase 8 or caspase 10 (Kischkel et al. 1995, Orrenius et al. 2003, Circu and Aw 2008). A complex consisting of the proteins Fas associated death domain (FADD) together with caspase 8 (FLICE) or caspase 10 (FLICE2), and FLICE-like inhibitory protein (cFLIP) initiates the extrinsic pathway (Tummers and Green 2017). Low levels of cFLIP enhance apoptotic signaling by activating caspase 8, whereas high levels of cFLIP inhibit apoptosis. Activated caspase 8 initiates apoptosis by cleaving effector caspases caspase 3 and caspase 7 (Tummers and Green 2017), whereas in other cell types there is cross talk to the intrinsic pathway (Orrenius et al. 2003). Caspase 8 can also initiate necroptosis by recruitment of other factors to the dissociated FADD-caspase 8 complex (reviewed in (Tummers and Green 2017)).

In intrinsic mitochondria-mediated apoptosis, effector caspases are activated after a signaling cascade. It can be triggered by intracellular stress conditions, including DNA damage, oxidative stress, cytosolic Ca^{2+} overload, endoplasmic reticulum stress, and mild excitotoxicity (reviewed by Galluzzi et al. 2012). Both pro-apoptotic and anti-apoptotic signals gather at the mitochondria which cause permeabilization of the outer mitochondrial membrane (MOMP) caused by pore formation of Bcl-2 family proteins Bax and Bak, or membrane rupture due to opening of the permeability transition pore (PTP) at the inner membrane (Galluzzi et al. 2012). This is followed by release of cytochrome c and second mitochondria-derived activator of caspases (SMAC/Diablo) resulting in formation of the apoptosome complex which triggers a proteolytic cascade with ATP-dependent cleavage of procaspase 9 which signals downstream cleavage/activation of effector caspases -3 and -6/7 (Circu and Aw 2008). Also, the intrinsic pathway can initiate caspase independent apoptosis through mitochondrial release of apoptosis inducing factor (AIF) which translocates to the nucleus and induces chromatin condensation and DNA fragmentation (Orrenius et al. 2003).

1.4.2 Glutathione and cell death

Depletion of glutathione is correlated with the induction of apoptosis (Circu and Aw 2008, Jeong and Joo 2016). The nucleus, endoplasmic reticulum and mitochondria have their own pools of glutathione and mitochondrial glutathione status is especially important in protecting mitochondrial DNA. Mitochondrial DNA is a critical cellular target for ROS due to the large amount of ROS generated through mitochondrial oxidative phosphorylation. In addition, mitochondrial DNA has an open circular structure, and DNA damage persists longer on mitochondrial DNA than nuclear DNA (Yakes and Van Houten 1997). Studies have described a positive correlation between decrease in mitochondrial glutathione and mitochondrial DNA damage, and that extensive mitochondrial DNA damage induces apoptosis (Circu and Aw 2008).

Both intrinsic and extrinsic apoptosis can be activated after depletion of glutathione (Franco and Cidlowski 2009). For instance, release of cytochrome c from the mitochondria is triggered by glutathione depletion, and it is proposed that depletion of cytosolic glutathione levels is needed for oxidation of the released cytochrome c and its pro-apoptotic activation (Franco and Cidlowski 2009). Another mechanism in regulating cell death and a critical regulator of cell signaling is post-translational modification of proteins through S-glutathionylation (also named S-glutathiolation), a reversible process where a disulfide bond

is established between a protein cysteine and GSSG. This modification protects against the irreversible oxidation of cysteine and is also a critical regulator of apoptosis (Circu and Aw 2008, Franco and Cidlowski 2009). Deglutathionylation removes disulfide bonds from gluthionylated cysteines and this process is an acute redox-sensitive regulatory step performed by redox proteins like thioredoxin and glutaredoxin (Grx) (Sykes et al. 2007). It has been shown that cleavage of caspase 3 as a result of the recruitment of the death domain FADD by TNF-α was inversely correlated with caspase 3 S-glutathionylation, and that increased activity of glutaredoxin was responsible for the deglutathionylation of caspase 3 and its activation (Pan and Berk 2007). It is also reported that the degree of glutathione depletion, paralleled by the levels of protein S-glutathionylation, may have a causal role in increasing calcium levels and cell death, and that S-glutathionylation works as a molecular linker between calcium and redox signaling (Frosali et al. 2009). These authors suggest that mitochondrial calcium increase could be responsible for apoptosis while cytoplasmic calcium overload could be associated with induction of necrosis (Frosali et al. 2009).

1.5 Glutamate and excitotoxicity

Lucas and Newhouse first noticed that injected glutamate caused degeneration of the inner layers of the retina in mice (Lucas and Newhouse 1957). Later, Olney was the first to use the term "glutamate excitotoxicity" to describe the ability of glutamate to cause degeneration of neurons (Olney 1969). The term is used to describe cell death in neurons due to excessive release of glutamate and overstimulation of *N*-methyl-D-aspartate (NMDA) receptors. Glutamate-induced neuronal death can result in apoptosis and necrosis both *in vitro* and *in vivo* (Ankarcrona et al. 1995, Yuan et al. 2003). Excess glutamate is implicated in various neurological disorders like AD, MS, ALS, HD, and PD, as well as the cause of neuronal death in pathological insults like traumatic brain or spinal cord injuries and stroke (Lipton and Rosenberg 1994, Lau and Tymianski 2010, Kostandy 2012).

1.5.1 Glutamate and glutamate receptors

Glutamate is the principal excitatory neurotransmitter in the mammalian nervous system, implicated in many neurological functions in the central nervous system, including learning, memory, cognitive functions, movement and sensation (Lipton and Rosenberg 1994). The glutamate system is crucial in generation of synaptic connections in early neuronal development, synapse maintenance and synaptic plasticity. The activity of the glutamate

system and its receptors determine how many neurons and the number of connections that are formed (Konradi and Heckers 2003).

The postsynaptic effects of glutamate are mediated by two groups of glutamate receptors (GluRs), the metabotropic receptors (mGluRs) and the ionotropic receptors (Ozawa et al. 1998). The mGluRs are single-peptide seven-transmembrane spanning proteins using G-protein as a transduction molecule, but G-protein-independent signaling may also occur (Heuss et al. 1999). They are divided into three groups (I, II, and III) and modulate synaptic transmission (reviewed in (Kostandy 2012)). The ionotropic receptors are divided into three types, including NMDA receptors, α-amino-3-hydroxy-5-methylisoxale-4-propionate (AMPA), and kainate receptors, and all subfamilies share common voltage-gated ion channel functions (Willard and Koochekpour 2013). AMPA receptors are composed of four subunits (GluR1-4), while the kainate receptors are made up of subunits from GluR5-7 and KAI-2. Both are activated by glutamate and are permeable to cation influx, sometimes Ca^{2+} (not the kaianate receptors) (Lau and Tymianski 2010).

The NMDA receptors have received most attention, due to their involvement in various processes, from learning and memory to neurodegeneration (Lau and Tymianski 2010, Paoletti et al. 2013). The channel of the NMDA receptor is composed of four subunits from three different gene families, NR1, NR2A-D, and NR3A-B. The typical NMDA receptor is made up of two NR1 subunits and two NR2 subunits. It requires the binding of both glutamate and glycine to their respective agonist domains for activation and allows flux of cations, preferably Ca^{2+}, but also others like Na^{+} and K^{+} when activated. Normally, Mg^{2+} is bound inside the ionic channel and blocks ionic flow even when the channel is activated by ligands, but the affinity of Mg^{2+} for the binding site is reduced when the membrane is depolarized. Therefore, ionic flow through the channel is allowed upon binding of the ligands in addition to depolarization of the membrane. Both AMPA receptors and kaianate receptors are thought to be involved in regulating the fast excitation required to remove the Mg^{2+} block of nearby NMDA receptors (Lau and Tymianski 2010).

1.5.2 Excitotoxicity - NMDA receptor signaling and neurodegeneration

Excitotoxic cell death can be divided into two components, based on difference in time course and ionic dependence (Choi 1992). The first component refers to acute neuronal swelling only minutes after exposure due to influx of Na^{+} and Cl^{-}, and there is a chance of recovery from the swelling. The second component is termed delayed neuronal disintegration. It occurs hours

after exposure resulting from excess influx of Ca^{2+} and activation of cell death signaling pathways. Ca^{2+}overload through NMDA receptors results in loss of mitochondrial membrane potential, causing of opening of permeability transition pore (PTP) and release of cytochrome c, caspase activation, and ROS generation through mitochondrial uptake of Ca^{2+} (Tymianski et al. 1993, Orrenius et al. 2003, Duan et al. 2007).

There has, for a long time, been an agreement that glutamate-induced neurotoxicity is Ca^{2+} dependent (Choi 1985), but the neurodegenerative process in excitotoxicity has been found to be caused by source specific Ca^{2+} influx and not the degree of Ca^{2+} entry through NMDA receptors (Tymianski et al. 1993). However, the NMDA receptors have a dichotomous nature in also being important in physiological processes like learning and memory. A more understandable picture emerged when it was discovered that the localization of the NMDA receptor determines the consequence of NMDA signaling. Thus, in the *localization hypothesis* synaptic NMDA receptors have anti-apoptotic activity, whereas stimulation of extrasynaptic NMDA receptors results in loss of mitochondrial membrane potential and cell death (Hardingham et al. 2002). In mature neurons, the extrasynaptic sites are almost entirely NR2B-containing NMDA receptors while synaptic sites consist mostly of NR2A-containing NMDA receptors (*subtype hypothesis*), making NR2B a potential target for neuroprotection. It is proposed that increases in the number of NMDA receptors located extrasynaptically and excess levels of glutamate in the extracellular space, are two important pathological biochemical states involved in several neurological diseases, in addition to a decrease in survival-promoting synaptic NMDA receptors (Bading 2017). Proximity of extrasynaptic NMDA receptors to mitochondria may be one reason for the toxicity of these receptors because mitochondria are exposed to high and damaging Ca^{2+} rises compared to mitochondria kept at a distance from synaptic NDMA receptors due to the postsynaptic scaffold, and the spine structure (Bading 2017). In addition, NMDA receptor-mediated Ca^{2+} influx activates NO production through a structural link between the neuronal NO synthase (nNOS) and NMDA receptors (Sattler et al. 1999). NO has by itself a number of intracellular targets, like interaction with glyceraldehyde 3-phosphate dehydrogenase (GADPH) (Lau and Tymianski 2010), but it also interacts with superoxide to form the strong oxidant peroxynitrite. Further, it is proposed that NO is involved in mitochondrial dysfunction by reaching mitochondria close in proximity to extrasynaptic NMDA receptors at high and damaging concentrations (Bading 2017). Thus, targeting extrasynaptic NMDA receptor signaling in neurodegenerations has gained attention as an interesting therapeutic goal (reviewed in (Bading 2017)).

1.6 Estrogen neuroprotection

The natural estrogen hormone in mammals is 17β-estradiol. In most vertebrate species, it is synthesized from the aromatization of androgens by cytochrome P450 aromatase in the gonads and in the brain (Simpson et al. 1994). Estrogens are important in reproductive processes and sexual behavior. In addition, numerous studies have revealed that estrogens exert non-reproductive actions on many organs and in physiological systems. It was early discovered that steroid hormones affect developmental patterns of neurites *in vitro* (Toran-Allerand 1976) and influence brain function (McEwen 1980). Today it is known that estrogen has important roles throughout the brain in different regions like learning and memory, excitability and seizures, neuroprotection, addiction, blood pressure, pain, motor coordination and fine motor skills (McEwen and Milner 2017). The neuroprotective properties of estrogen were discovered in the 1990s when estrogen replacement therapy in older women was associated with decreased risk of AD (Henderson et al. 1994, Paganini-Hill and Henderson 1994). This was followed by several *in vitro* and *in vivo* studies showing that 17β-estradiol was neuroprotective against the neurodegenerative effects of stroke (Shi et al. 1997, Simpkins et al. 1997b, Weaver et al. 1997), and AD (Green et al. 1996, Simpkins et al. 1997a). In the same period, it was discovered that its stereoisomer 17α-estradiol which has low estrogenic activity (Kuiper et al. 1997), was equally neuroprotective as 17β-estradiol (Behl et al. 1997, Green et al. 1997a, Green et al. 1997b) (Figure 3).

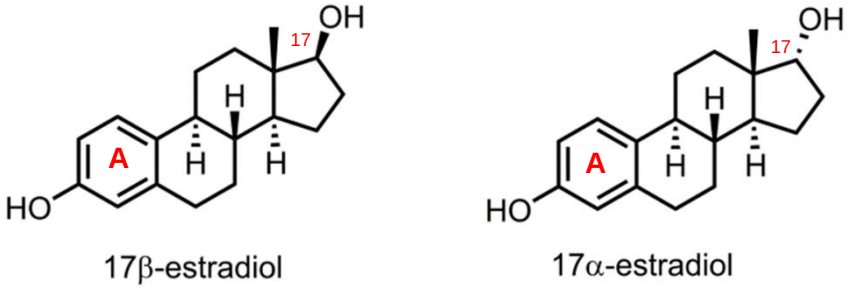

Figure 3 *Chemical structures of the natural estrogen 17β-estradiol and its isomer 17α-estradiol. The hydroxyl group in the phenolic A ring is critical for neuroprotection and the molecules differ in their stereochemistry at position 17 (indicated in red).*

This discovery opened the door for that the neuroprotective activity of estrogen could be mediated independently of estrogen receptors (ERs), and it was demonstrated the antioxidant activity of the estrogen molecule was found to be dependent on the hydroxyl group in the C3 position on the A ring (Behl et al. 1997, Green et al. 1997b). However, it is also shown that 17β-estradiol exerts protection and survival of neurons through its receptors ERα, ERβ or the G protein-coupled estrogen receptor (GPER) (Dubal et al. 2001, Wang et al. 2001, Miller et al. 2005, Lebesgue et al. 2009, Liu et al. 2011, Liu and Zhao 2013), revealing many possible mechanisms and a complex picture behind estrogen neuroprotection.

Due to increased risk of stroke and dementia associated with menopause in women and the neuroprotective properties of estrogen, The Women's Health Initiative (WHI) performed a large-scale hormone replacement therapy (HRT) study to assess major health benefits and risks of estrogen plus progestin in healthy postmenopausal women. However, the trial was stopped prematurely when overall health risks exceeded benefits from use of HRT, with invasive breast cancer as adverse outcome in addition to increased risk of stroke (Rossouw et al. 2002, Wassertheil-Smoller et al. 2003). Other studies also found increased ischemic stroke damage in an animal model with 17β-estradiol (Carswell et al. 2004, Bingham et al. 2005, Theodorsson and Theodorsson 2005). Furthermore, the Women's Health Initiative reported increased risk of dementia in women aged 65 years or older (Shumaker et al. 2003). Today it is accepted that 17β-estradiol is a conditional neuroprotective agent (Engler-Chiurazzi et al. 2016).

1.6.1 Mechanisms of estrogen neuroprotection

17β-Estradiol protects neurons through four proposed mechanisms that involve genomic signaling, rapid non-genomic signaling, antioxidant actions, and regulation of mitochondrial bioenergetics (reviewed in (Scott et al. 2012)). Genomic signaling through ERs, particularly ERα, is suggested to mediate many of the effects. ERα was discovered first and it took many years before ERβ was cloned (Kuiper et al. 1996). ERα and ERβ are proteins which translocate to the nucleus and bind to estrogen response elements (ERE) in the form of a transcription complex upon binding of a lipophilic estrogen ligand, regulating transcription of genes. A non-classical mechanism involves indirect binding through transcription factors like AP-1 (Levin 2005). Though many studies have demonstrated the importance of ERα in neuroprotection, the picture is, however, complex as the protection is dependent on cell types. For instance, 17β-estradiol was shown to be neuroprotective in microglia-specific ERα

knockout mice, but not in neuron-specific ERα knockout mice against cerebral ischemia (Elzer et al. 2010). Rapid non-genomic signaling occurs through intracellular signaling pathways through MAPK/ERK or phosphatidylinositol-3-kinase/Akt (Alexaki et al. 2004, Alexaki et al. 2006) mediated by binding of estrogen to plasma membrane-associated receptors, either GPER or a caveolin-associated form of ERα (Hamilton et al. 2017). Both ERα and ERβ are shown to be located at the plasma membrane of neurons in various brain regions and are proposed to play a key role in mediating rapid non-genomic effects in neurons (Scott et al. 2012). Interestingly, a truncated ER receptor variant, ER-α36 located on the plasma membrane discovered in 2005 (Wang et al. 2005), is also reported to be involved in estrogen neuroprotection through rapid signaling of MAPK/ERK and phosphatidylinositol-3-kinase (Han et al. 2015). In addition, a Gq-coupled membrane ER (Gq-mER) exists which has a role in sexual behavior, and like ERα, rapid membrane initiated signaling triggers a signal transduction cascade involving phospholipase C, protein kinase C, protein kinase A, as well as phosphatidylinositol-3-kinase (Conde et al. 2016). Moreover, crosstalk with classical ERs and GPER within the cell is postulated to modulate signaling in five different scenarios which complicates estrogen-meditated signaling even further (Hadjimarkou and Vasudevan 2017).

In regard to glutamate-induced neurotoxicity, it is suggested that ERs are involved in the neuroprotection of estrogen through mGluRs, inhibition of the release of glutamate, enhanced glutamate transporter expression and/or through modulating NMDA receptors (Lan et al. 2014). However, estrogens also potentiate calcium influx of L-type voltage-gated Ca^{2+} channels which mediate rapid intracellular signaling linked to events independent of ER interactions like modulation of synaptic plasticity, neuroprotection, and memory formation (Sarkar et al. 2008).

Mitochondrial dysfunction is most often implicated in neuronal damage and 17β-estradiol preserve mitochondrial function by maintenance of sufficient neuronal ATP, and by effects on ROS production, mitochondrial apoptotic factors, and antioxidant mechanisms (Borras et al. 2010, Scott et al. 2012). The antioxidant function is due to its phenolic A ring as a direct hydroxyl scavenger in an ER-independent manner (Green et al. 1997b, Prokai et al. 2003), but ERα has also been implicated in its antioxidant activity by attenuating superoxide anion production and NADPH oxidase activation (Scott et al. 2012). However, at concentrations below 500 nM it is unlikely that estrogens act as a direct reductant/antioxidant, but rather through ligand binding (Santanam et al. 1998, Moosmann and Behl 1999, Borras et al. 2010).

Estrogen neuroprotection is dependent on glutathione levels. For instance, an ER-independent synergistic interaction between glutathione and 17β-estradiol has been demonstrated, where glutathione had to be added for full protection against β-amyloid-induced toxicity (Gridley et al. 1998). Also, it is shown that 17β-estradiol reduces glutathione synthesis and endogenous glutathione levels through a pretreatment period in cerebellar granule neurons, thus hampering its ability to protect against glutamate-induced toxicity (Fallgren et al. 2007).

1.6.2 17α-Estradiol and non-feminizing estrogens

17α-Estradiol was among the first estrogen molecules with low estrogen activity discovered with neuroprotective properties. It has two times and 10 times lower affinity for ERα and ERβ (Kuiper et al. 1997), and has little affinity for GPER below concentration at 10 μM (Thomas et al. 2005). Due to its potential, a phase I clinical study with oral 17α-estradiol (sodium sulfate conjugate) has been conducted, and (Dykens et al. 2005) revealed positive and successful pharmacokinetic data from this study. 17α-Estradiol protects neurons through several mechanisms including through its hydroxyl group in the C3 position on the A ring, preservation of membrane integrity by terminating lipid peroxidation chain reactions in cell membranes, and/or through activation of multiple signaling proteins like MAPKs including B-Raf and ERK (reviewed in (Dykens et al. 2005)). However, it is also demonstrated that 17α-estradiol does not exert protection in some models (Weaver et al. 1997, Chae et al. 2001). Although it is reported that 17α-estradiol is the hormonally inactive isomer of 17β-estradiol, it is found in low concentration of animals (Robinson et al. 1973, Gwilliam et al. 1974) and in low concentrations in the urine and serum in humans (Hobe et al. 2002, Moos et al. 2009). Also, 17α-estradiol is found to be biologically active in the uterine tissue (Perusquia and Navarrete 2005).

In general, it is stated that neuroprotection by estrogens is to a certain degree mediated independently of ERs. As the benefits of estrogen replacement treatment has been controversial due to side effects of chronic estrogen treatment, many estrogen analogs have been developed with the goal of finding analogs that exert neuroprotective effects avoiding the side effects of chronic estrogen treatment (Simpkins et al. 2013, Petrone et al. 2014). Non-feminizing estradiol analogs with no or reduced ER binding are proposed to exert neuroprotection equally or more potent than 17β-estradiol, and part of the neuroprotection of these non-feminizing estrogens is due to enhancement in redox potential (Simpkins et al. 2013). Thus, these efforts of synthesizing estrogen analogs are motivated by the potential to

use non-feminizing estrogens for clinical application (Simpkins et al. 2013, Petrone et al. 2014).

2. Aims of the study

The balance between the endogenous antioxidant system and ROS generation determines the degree a cell can protect itself during an oxidative insult. The endogenous antioxidant glutathione is part of the main defense and is important in maintaining the intracellular redox balance. Despite estradiol's proposed potential as neuroprotector, its acute protection against glutamate-induced toxicity in cerebellar granule neurons was lost after a pre-incubation period since the pretreatment period reduced levels of glutathione. Pretreatment with both 17α-estradiol and 17β-estradiol reduced the neuroprotection (Fallgren et al. 2007).

The general aim of this study was therefore to investigate how 17α-estradiol and different estradiol analogs may act differently as neuroprotectors due to possible interference with endogenous glutathione, with potential implication for use of estrogens as neuroprotectors. Serum-deprived PC12 cells and glutamate-induced toxicity in chicken cerebellar granule neurons were applied as models to challenge the antioxidant defense and to induce cell death.

Specific aims are:

1) Investigate how 17α-estradiol works as a neuroprotector in serum-deprived PC12 cells; with emphasis on molecular mechanisms of how the steroid interferes with endogenous production of glutathione (papers I and III).
2) Finding an estradiol analog with low estrogen activity (paper II) and investigate if modifying estradiol results in molecules with better properties as a neuroprotector regarding glutathione regulation in serum-deprived PC12 cells (paper III).
3) Explore mechanisms behind the neuroprotective effects of 17α-estradiol in different time windows in cerebellar granule neurons (paper IV).

3. Summary of papers

Paper I: 17α-Estradiol down-regulates glutathione synthesis in serum deprived PC-12 cells.

Rakkestad K.E[1], Sørvik I.B[1], Øverby G.R, Debernard K.A.B, Mathisen G.H, Paulsen R.E.

[1]These authors contributed equally

Free radical research (2014)

Estrogen has neuroprotective properties, and its stereoisomer 17α-estradiol with low estrogen activity is equally neuroprotective as 17β-estradiol. However, estradiol may have an impact on glutathione levels. This paper evaluated the neuroprotective properties of an antioxidant concentration of 17α-estradiol (10 μM) and antioxidant vitamins in combination (C/E) in serum-deprived rat pheochromocytoma cell line PC12. While vitamins C/E tended to protect against serum deprivation-induced cell death, 17α-estradiol further enhanced the deprivation-induced cell death at time points 24 h and 48 h. We confirmed that glutathione is partly involved in serum-deprived toxicity since the cysteine precursor with potential to restore glutathione, N-acetylcysteine, reduced the cell death. Also, endogenous glutathione levels decreased with serum deprivation. Further, 17α-estradiol reduced both glutathione levels and the promoter activity of the catalytic subunit of the rate-limiting enzyme in glutathione synthesis, glutamate cysteine ligase (GCLC), in serum-deprived cells. The decreased glutathione levels with 17α-estradiol were partly due to reduction in deprivation-induced c-Fos protein levels. Protein levels of the transcription factor Nrf2 were not affected by 17α-estradiol. c-Fos is part of the AP-1 transcription factor complex which binds to the AP-1 response element in the GCLC promoter, and AP-1 binding can contribute to increased production of glutathione during an oxidative insult. Hence, overexpression with AP-1, but not Nrf2, restored some of the lost GCLC promoter activity induced by 17α-estradiol. These results suggest that 17α-estradiol may have a long-term negative effect on endogenous glutathione levels due to down-regulation of glutathione synthesis during serum deprivation.

Paper II: Synthesis and biological evaluations of new analogs of 2-methoxyestradiol: Inhibitors of tubulin and angiogenesis.

Solum E.J, Cheng J-J, Sørvik I.B, Paulsen R.E, Vik A; Hansen T.V.

European Journal of Medicinal Chemistry (2014)

2-Methoxyestradiol (2-ME) is a natural metabolite of 17β-estradiol, and a potent anti-cancer agent with anti-vascular effects and anti-angiogenetic activities. Due to its potential, several new 2-ME analogs with anti-cancer activities have been synthesized. In this study 15 new 2-ME analogs were prepared with either a pyridine, a quinoline or an isoquinoline ring in the C-17 position of 2-ME. Effects on the synthesis, cytotoxicity, inhibition of tubulin polymerization, and anti-angiogenetic were evaluated for all 15 compounds. In addition, estrogen activity of five analogs at 10 μM concentration was tested. The position of the nitrogen atom in the heterocyclic ring was found to be important for potent inhibition of angiogenesis and tubulin polymerization for the compounds. The most potent inhibitors had a substitution with a 6 iosquinoline (6iq) ring in the 17-position of the steroid ring. Also, all compounds tested had low estrogen activity. The most potent compounds in this study provide new information about further structural-activity relationship studies towards the development of new anti-cancer agents.

Paper III: Novel estrogen analogs with improved neuroprotective properties in serum-deprived PC12 cells.

Sørvik I.B, Solum E.J, Hansen T.V, Paulsen R.E.

Manuscript (2017)

Non-feminizing estradiol analogs with no or reduced estrogen receptor (ER) binding are proposed to exert neuroprotection with the benefit of avoiding side effects of chronic estrogen treatment. The main objective in paper III was to compare neuroprotective properties of 17β-estradiol and 2-methoxyestradiol (2-ME) analogs together with 17α-estradiol, and an antagonist on G protein-coupled estrogen receptor (GPER) G36 in serum-deprived rat pheochromocytoma PC12 cells with both low (1 nM) and high (10 μM) concentrations. All compounds tested had low estrogen activity compared to 17β-estradiol. None of the compounds protected against deprivation-induced cell death at 10 μM, but both 17α-estradiol and 2-ME enhanced the cell death. In addition, they both decreased intracellular glutathione levels. At 1 nM, eight out of 12 compounds protected against deprivation-induced cell death,

including 17α-estradiol, G36 and 2-ME. The enhanced cell death and the reduced glutathione levels with 10 µM 17α-estradiol were linked to increased protein levels of activated p38 mitogen activated protein kinase (p-p38). However, the two 2-ME analogs modified with a 6 isoquinoline moiety (6iq) both protected against the deprivation-induced cell death at 1 nM, and enhanced the promoter activity of the catalytic subunit of the rate-limiting enzyme in glutathione synthesis, glutamate cysteine ligase (GCLC) at 10 µM. In addition, GCLC protein levels and Nrf2 protein levels increased with the novel 2-ME analogs. Thus, 17α-estradiol and 2-ME are less suitable as experimental neuroprotectors due to interference with glutathione at 10 µM, but modifying 2-ME may give the steroid ability to positively interfere with glutathione regulation.

Paper IV: High and low concentration of 17α-estradiol protect cerebellar granule neurons in different time windows

Sørvik I.B, Paulsen R.E.

Biochemical and Biophysical Research Communications (2017)

Both 17β-estradiol and its hormonally inactive isomer 17α-estradiol are regarded as neuroprotectors. However, it is previously reported that a pretreatment period with both steroids at 10 µM concentration abolished their acute neuroprotection of cultured cerebellar granule neurons (CGNs) from glutamate-induced cell death. In this study, we used 17α-estradiol as a neuroprotector in both low (1 nM) and high (10 µM) concentrations in chicken CGNs against glutamate-induced cell death. We reveal that 10 µM, but not 1 nM, is neuroprotective when administered together with glutamate (acute). However, a 24 h pretreatment period with 1 nM protects against cell death, whereas 10 µM fails to protect. Calcium response during glutamate exposure seemed partly implicated with the non-protective treatments as the protective treatments had lower calcium response. Further, protein level of the N-methyl-ᴅ-aspartate receptor subunit NR2B was upregulated and glutathione levels were reduced after pretreatment with 10 µM. Thus, these results suggest that17α-estradiol has a time and concentration dependent ability to protect against glutamate excitotoxicity.

4. Methodological considerations

4.1 Cell models

There is a significant distance to extrapolate *in vitro* results to *in vivo* conditions, but cell lines and cultured neurons are useful tools to study molecular mechanisms. The pheochromocytoma PC12 cell line and chicken cerebellar granule neurons were used as *in vitro* models in this thesis.

4.1.1 The PC12 cell line

The clonal neuroendocrine cell line PC12 was established from transplantable pheochromocytoma, a tumor arising from the rat adrenal medulla (Greene and Tischler 1976). PC12 cells differentiate into a sympathetic neuron and extend neurites in response to nerve growth factor (NGF). The cells synthesize and store catecholamines (dopamine and norepinephrine) and offers advantages over primary cultured neurons through the ability to provide high throughput and retention of a mature phenotype (Kinarivala et al. 2017). Due to its resemblance to neurons and neuron-like properties, it is a widely used cell line as a model of neuronal differentiation, neurodegenerative diseases, and cell apoptosis. Neurodegenerative diseases such as Parkinson's disease can be modeled through exposure with the neurotoxin 1-methyl-4-phenylpyridinium (MPP+), 6-hydroxydopamine (6-OHDA), rotenone, or paraquat (Grau and Greene 2012), AD through injury by β-amyloid peptide (1-42) (Wei et al. 2000), and ischemic stroke through oxygen and glucose deprivation (Tabakman et al. 2002) or hypoxic/ischemic induced conditions (Jung et al. 2008) in PC12 cells. Further, estrogen is demonstrated to protect against modeled Parkinson's disease, AD and hypoxic/ischemic conditions in PC12 cells, making the PC12 cell line an interesting model to study neuroprotective effects of estrogens (Habauzit et al. 2011).

4.1.2 PC12 cells as a model for neuronal cell death

Serum-free conditions induce apoptosis in cultured PC12 cells (Greene 1978, Batistatou and Greene 1991, Rukenstein et al. 1991) by activating caspases, particularly caspase-2 (Stefanis et al. 1998). Thus, serum-deprived PC12 cells do not undergo mitosis and 99% of the cells die within 2-3 weeks. Activation of caspase-2 can sometimes be triggered in the absence of activation of both the intrinsic and extrinsic pathways of apoptosis or it can be linked to both (Kim and Dass 2012). NGF rescues PC12 cells from the deprivation-induced cell death,

suggesting that like the sympathetic neurons, PC12 cells requires NGF for survival and that serum substitutes for this requirement (Greene 1978). Serum-deprived PC12 cells are therefore a useful model to study neuronal cell death. Already within three hours of serum withdrawal before any morphological signs of cell death, PC12 cells have endonuclease activity which leads to internucleosomal cleavage of their cellular DNA (Batistatou and Greene 1991). The DNA fragmentation can be inhibited by NGF or serum.

Depletion of intracellular glutathione levels triggers programmed cell death in PC12 cells (Froissard et al. 1997). PC12 cells are reported to have low intracellular glutathione levels and are more susceptible to toxic insults than other cell types containing higher levels of antioxidant defenses (Calderon et al. 1999), suggesting that survival of PC12 cells is linked to intracellular levels of glutathione. Also, a rapid increase in oxidized glutathione resulting in cellular redox imbalance can induce apoptosis in undifferentiated PC12 cells independently of ROS production (Pias and Aw 2002). Since serum deprivation is challenging the antioxidant defense, deprived PC12 cells were used as model to investigate the neuroprotective properties of 17α-estradiol and other estradiol analogs through possible interference with glutathione regulation during deprivation-induced cell death (papers I and III).

Previous studies report that undifferentiated PC12 cells do not express ERα, but ERβ, while differentiated neuronal PC12 cells express ERα and ERβ (Gollapudi and Oblinger 1999, Gelinas et al. 2004). Thus, 17β-estradiol is shown to protect through receptor-dependent pathways (Chae et al. 2001) in PC12 cells. Also, protective effects of estrogen on cell survival in transfected PC12 cells with ERα (Gollapudi and Oblinger 1999), as well as downstream activation of pro-survival kinases through membrane ERs after serum deprivation in PC12 cells (Alexaki et al. 2006) is demonstrated. When our undifferentiated PC12 cells were transfected with a plasmid containing ERE coupled to a luciferase reporter (ERE-luc), no activity was detected when treated with 17β-estradiol, confirming that our cells do not contain classical ERs (paper I).

Though PC12 cells are regarded as a useful *in vitro* model to study effects on neurons, they have the disadvantage of not being real neurons. Also, a recent report has revealed that passage variation can result in false-positive/false-negative identification of neuroprotective compounds (Kinarivala et al. 2017). Thus, undifferentiated PC12 cells with high passage number were reported to be less sensitive to insults like serum deprivation or etoposide treatment. These findings reflect the observed deprivation-induced cell death in our PC12

cells as the response of serum withdrawal has varied from 2% to 30% and probably also reflects the variations in data within the other assays. The PC12 cells in our lab are used from passage 4-5 to 50. Efficient removal of serum is another critical factor that can give rise to variations. In addition, optimizing protocols for assays PC12 cells have offered some challenge since the cells have a tendency to detach during serum deprivation and non-deprived cells increase in number due to cell division.

4.1.3 Primary cultures of cerebellar granule neurons from chicken

Cerebellar granule neurons (CGNs) constitute the largest homogenous neuronal population of the mammalian brain (Contestabile 2002). In culture, they morphologically differentiate and develop into mature neurons, thus resembling *in vivo* conditions with neuritic network, expression of excitatory amino acid receptors (e.g. NMDA receptors), and synthesis and release glutamate (Gallo et al. 1982). CGNs from young mice and rats represent *in vitro* models that have been widely used to study mechanisms implicated in survival/apoptosis, neuroprotection, neuronal development, function and pathology, including glutamate excitotoxicity (Contestabile 2002, Vaudry et al. 2003). Neurodegenerative diseases and pathological injury seldom occur in the mammalian cerebellum *in vivo*, but since conditions that do occur *in vivo* can be mimicked in CGNs cultures, CGNs have acquired a position as a reliable model to study neurons (Contestabile 2002).

Survival of rat and mice granule cells in culture require depolarizing culturing conditions (25 mM K^+) that mimic the physiological natural conditions in the cerebellum (Gallo et al. 1987), and the model requires use of animal facilities. Chicken (*Gallus gallus*) are birds (Aves), but the histological architecture of the cerebellum is highly conserved in all vertebrae. Granule cells of the cerebellum of chicken, first cultured by our research group, represent an alternative culture model, with the benefit of avoiding animal facilities, being inexpensive, and requiring only 5 mM K^+ (Jacobs et al. 2006). Cultured chicken CGNs have a yield of 80% neurons. They mature faster than rat CGNs, and develop excitotoxicity at 3 days *in vitro* compared to 7 days *in vitro* with cultures of rat CGNs. Chicken neurons have generally higher basal cell death compared to rat cultures, but rat and chicken CGNs respond similarly to glutamate excitotoxicity with ROS production and activation of caspase-3 (Jacobs et al. 2006).

Cultured chicken CGNs were used as model to mimic glutamate excitotoxicity at 4 days *in vitro*, using 100 µM glutamate which induces apoptosis in cultured neurons (Cheung et al. 1998). Neuroprotective properties of 17α-estradiol using low and high concentrations in

different time windows were addressed in regard to calcium response, ROS production, and glutathione after administration of glutamate (paper IV).

4.2 Glutathione measurements

Intracellular glutathione status determines the cellular ability to resist a toxic challenge. Depletion in total glutathione level in the cell is either due to reduced synthesis and/or transport of accumulated GSSG out of the cell during severe oxidative stress. Decrease in total glutathione level does not, however, give information about the GSH/GSSG ratio which indicates the redox state in the cell. In spectrophotometric or fluorescent detection, direct labeling of the cysteine thiol or other reactive amino acids moiety is required for quantification of glutathione (Hansen and Harris 2015). Methods that quantify both reduced and oxidized glutathione (GSH/GSSG) like high-performance liquid chromatography (HPLC) with electrochemical detection can be used to determine a shift in the ratio (Rebrin et al. 2007, Yap et al. 2010).

Glutathione levels were measured in the PC12 cells (papers I and III) and in the CGNs (paper IV) with a Glutathione Assay kit from Sigma or with the cell-permeant fluorescent probe monochlorobimane (mBCl). The assay kit uses a kinetic assay to measure the level of total glutathione (GSSG+GSH) in a biological sample. The mBCl probe detects thiol groups. Non-enzymatically mBCl interacts with reduced GSH and has only minor interaction with oxidized GSSG, but in the presence of GST it reacts specifically with reduced GSH to form a fluorescent conjugate (Stevenson et al. 2002). The dye has one drawback due to limitations of the GST isoenzyme present in the cells. Hence, conjugation of mBCl and GSH is catalyzed by GSTμ and to some degree by GSTα, but it is a poor substrate for the GSTπ isoenzyme in comparison (Ublacker et al. 1991). PC12 cells are shown to express GSTμ (Nur et al. 2000).

4.3 ROS detection

Both deprivation-induced cell death in the PC12 cells and glutamate-induced toxicity in the CGNs are insults that lead to increases in ROS production. For detection of reactive species DHR (dihydrorhodamine 123) was used (papers III and IV). DHR oxidizes to the fluorescent product rhodamine in the presence of ROS in a reaction catalyzed by a peroxidase or a similar catalyst. It has been reported to detect hydrogen peroxide formation (Henderson and Chappell 1993) as well as being a sensitive and efficient trap for peroxynitrite (Kooy et al. 1994), both generated from superoxide.

5. Discussion of results

Figure 4 illustrates the main results in this thesis. The results are further discussed in sections 5.1-5.6.

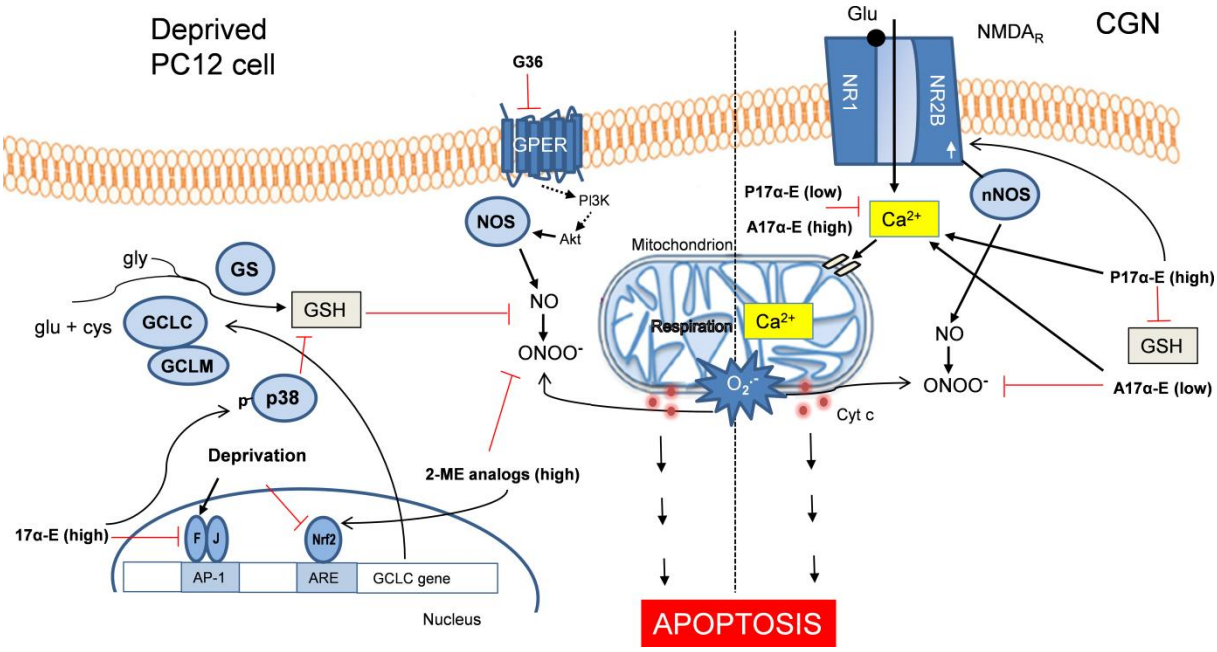

Figure 4 ***Deprived PC12 cell*** *(left panel): Deprivation induces apoptosis in PC12 cells. Oxidative stress causes increased synthesis of GSH due to consumption of glutathione (GSH). GCLC catalyzes the formation of glutamate (glu) and cysteine (cys) while GS adds glycine (gly). The stress-induced response by deprivation up-regulates transcription of the GCLC gene through AP-1 by enhancing c-Fos (F) which dimerizes with c-Jun (J) in the AP-1 protein complex. 17α-Estradiol (17α-E (high)) down-regulates the deprivation-induced GCLC promoter activity via reduced c-Fos level and decreases GSH level trough increased level of p-p38. 2-ME analogs (high) increase the deprivation-induced GCLC promoter activity and GCLC level through Nrf2.The GPER antagonist G36 decreases deprivation-induced ROS generation through GPER with reduced peroxynitrite formation via inhibition of NO formation and superoxide formation.* ***Cerebellar granule neuron (CGN)*** *(right panel): Glutamate (Glu) causes Ca^{2+} overload through NMDA receptors resulting in loss of mitochondrial membrane potential and release of cytochrome c (Cyt c). NMDA receptor mediated Ca^{2+} influx activates NO production through a structural link between NMDA receptor and nNOS. The protective treatments, pretreatment, P17α-E (low) and acute, A17α-E (high), reduce the glutamate-induced Ca^{2+}response, while A17α-E (low) and P17α-E (high) do not. A 24 h pretreatment period with P17α-E (high) results in enhanced NMDA receptor subunit NR2B level and decreased GSH level. High=10 µM, low= 1 nM. P=pretreatment (24 h prior to glutamate), A=acute treatment (together with glutamate).*

5.1 17α-Estradiol and glutathione

In paper I and III we show that an antioxidant concentration (10 µM) of 17α-estradiol enhances deprivation-induced cell death in PC12 cells. This was due to down-regulation of the deprivation-induced promoter activity of the catalytic subunit of the rate-limiting enzyme GCLC in glutathione synthesis by 17α-estradiol followed by decrease in glutathione levels during serum deprivation. Also, 17β-estradiol reduced the synthesis with both 1 nM and 10 µM concentrations. However, 1 nM of 17α-estradiol protects against deprivation-induced cell death and did not reduce the glutathione levels (paper III), which suggest that only an antioxidant concentration interferes with regulation of glutathione. Reduced protein level of the c-Fos transcription factor involved in binding to the AP-1 element in the GCLC promoter seemed implicated in the reduced synthesis as over-expression of AP-1 proteins restored the GCLC promoter activity in deprived cells treated with 10 µM 17α-estradiol (paper I). The AP-1 protein complex consists of homodimers of Jun family members, heterodimers of Jun and Fos, or cAMP response element-binding protein (CREB)/activating transcription factor family proteins (ATF). AP-1 has an important role in cell proliferation, differentiation, death, and survival. The regulation of AP-1 activity is, however, complex (further reviewed by (Shaulian and Karin 2002)).

Interestingly, NGF rescues PC12 cells from deprivation-induced cell death and it is shown that NGF induces expression of members of the AP-1 complex, *c-fos* and *c-jun* mRNA during serum deprivation (Morgan and Curran 1991). Others have reported persistent AP-1 DNA binding activity during serum deprivation with NGF, while deprivation alone induces transient increase in AP-1 DNA binding activity (Tong and Perez-Polo 1996). Specific components were shown to participate (e.g. c-Fos, c-Jun, Fra 2 and Jun B) in the increased AP-1 DNA binding by NGF. It was emphasized that transient up-regulation of AP-1 DNA binding activity after serum deprivation is a stress response by the cell machinery meant to protect and enhance survival of cells after loss of energy and oxidative homeostasis (Tong et al. 1999). Moreover, as AP-1 is involved in regulation of glutathione synthesis, it seems probable that this increased AP-1 activity results in increased levels of glutathione in PC12 cells as NGF is reported to protect PC12 cells from oxidative insults in PC12 cells through increased GCL activity and increased glutathione levels (Pan and Perez-Polo 1993). Further, Nrf2 was not involved the up-regulated GCLC promoter activity as protein level of Nrf2 was not affected by deprivation in paper I, and decreased in paper III. Together, these findings strongly suggest that AP-1 is involved in the up-regulated GCLC promoter activity during

serum deprivation in our PC12 cells and that 10 µM of 17α-estradiol interferes in this process. The observation in our PC12 cells, however, may not be functionally relevant in other cell types or environmental conditions. Thus, the AP-1 transcription factors can both induce and prevent apoptosis depending on induction of pro-apoptotic and anti-apoptotic target genes that decide the final outcome, but activation of AP-1 in cells that proliferate will further promote cell proliferation and survival (Shaulian and Karin 2002).

The p38 MAPK pathway is implicated in regulation of glutathione synthesis (see introduction), and the mechanism behind the reduced glutathione levels by 10 µM 17α-estradiol was further explored in paper III where we show that 10 µM 17α-estradiol increases activated p38 (p-p38) during serum deprivation. Moreover, when p38 was inhibited, the reduced glutathione levels with 17α-estradiol were restored. The protective concentration at 1 nM did not increase p-p38. As p38 is reported to increase or decrease glutathione synthesis and/or glutathione levels (Gu et al. 2016, Huseby et al. 2016, Koyani et al. 2016, Jeong et al. 2017), it is clear that the role of p38 is cell type and stimulus dependent. Interestingly, p38 and AP-1 induction can be linked together. Thus, 6-hydroxydopamine-induced cell death in PC12 cells was reported to be caused by degradation of myocyte enhancer factor 2 (MEF2D) and induced activation of p38 (Kim et al. 2011). MEF2 is important for neuronal survival and the isoform MEF2D is reported to play a critical role in dopaminergic neuronal death *in vivo* (Smith et al. 2006) making the PC12 cells a highly relevant model to study MEF2. Further, p38 can contribute to AP-1 induction through another isoform (MEF2C) (Han et al. 1997), and induction of AP-1 by pro-inflammatory cytokines and genotoxic stress is to a certain degree known to be mediated by the p38 MAPK pathway (Chang and Karin 2001). It would therefore be interesting to investigate the role of MEF2 and p38 on AP-1 induction in our PC12 cells to further explore how 17α-estradiol interferes with glutathione regulation.

In the chicken CGNs, pretreatment with 10 µM 17α-estradiol for 24 h reduced the glutathione levels (paper IV). This was, however, not due to reduced synthesis (results not shown), which was the case with 17β-estradiol in mice CGNs (Fallgren et al. 2007). Thus, the p38 MAPK pathway could be explored for reduced glutathione levels in the chicken CGNs.

5.2 Estrogen analogs and neuroprotection

Due to the ability of estrogen to mediate neuroprotection independently of ERs, efforts have been made in making non-feminizing estrogen analogs that may have the potential for clinical application (see introduction). Paper II, however, had another motivation for making estradiol analogs of the natural 17β-estradiol metabolite 2-ME. 2-ME is a potent anti-cancer agent in micromolar concentrations (Kumar et al. 2016) and several new 2-ME analogs have been synthesized due to its potential. Naturally, 2-ME is present in picomolar concentrations, but increases to nanomolar concentrations during late pregnancy (Berg et al. 1983). This suggests that 2-ME is not toxic in these concentration ranges. The aim of paper II was to find 2-ME analogs with effects on angiogenesis and tubulin polymerization and some of the analogs were tested as agonists on ERα since it is desirable to find an anti-cancer agent with low estrogen activity. Compounds 11f and 13e were found to be the most potent inhibitors with IC_{50} values in the lower micromolar range. The work on estrogen activity in the PC12 cells was performed in our lab and the inhibition assays were done in three different human cancer cell lines K562, OVCAR-3 and WM35. The readers are referred to Erratum for corrected presentation of analogs tested in paper II (Scientific paper I-IV).

In paper III, however, we report 2-ME analogs originally made with the purpose as anti-cancer agents, to have neuroprotective properties in PC12 cells. Thus, the most potent steroids 11f and 13e modified with a 6 iosquinoline (6iq) at position C17 in the steroid ring in paper II (named 2ME-11f-db and 2ME-13e in paper III, respectively), were found to be the ones with the most favorable properties in regard to glutathione regulation. They acted opposite of 17α-estradiol at 10 μM due to lack of enhancement in deprivation-induced cell death, increase in GCLC promoter activity and GCLC protein level, no reduction in glutathione levels, increase in Nrf2 protein (11f), and no increase in p-p38 protein level. Based on the results from paper II, the original plan was not to investigate 11f and 13e. During the period when the 2-ME analogs were tested for estrogen activity in paper II, these and other analogs were screened in deprived PC12 cells. We wanted to investigate if modification of the 17β-estradiol steroid and the 2-ME steroid could give the molecules improved neuroprotective properties compared to 17α-estradiol. The two estradiol analogs E-3py and E-4iq in paper III (not included in paper II) were hypothesized be the best candidates, but to our surprise, 2-ME and five other 2-ME analogs protected against deprivation-induced cell death at 1 nM concentration. In addition, only 2-ME enhanced the deprivation-induced cell death at 10 μM. The only difference between the 11f and 13e is the absence of the 16,17-double bond in 13e.We noticed that this

difference did not enhance the deprivation-induced cell death when the steroid was modified with 6iq as it did with the analogs modified with 3-pyridine (3py). Compounds 11f and 13e were therefore viewed as interesting. The PC12 cells were, however, not included in the cancer cell growth inhibition assay in paper II. As 11f and 13e had IC_{50}-values of 0.4 µM and 0.7 µM, respectively, they were slightly more active than 2-ME with IC_{50}-value of 0.8 µM in the K562 cell line. Preliminary results from MTT assay in our lab show that only 2-ME at 10 µM, but not 11f and 13e, reduces the viability in non-deprived PC12 cells, most likely because of growth inhibition. Also, none of the compounds presented in paper III induced cell death in non-deprived PC12 cells at either concentrations (1 nM and 10 µM), including 2-ME, with trypan blue exclusion. Even though 11f and 13e have neuroprotective properties in PC12 cells, they have also a potential as anti-cancer agents. These results demonstrate the importance of conducting studies in different cell types and different endpoints. Further studies are therefore needed in understanding more of the neuroprotective properties of such analogs. The hope is to find analogs with low estrogen activity and no effect on angiogenesis and tubulin, but paper III reveals that compounds with anti-cancer effects at high concentrations can have protective properties at low concentrations.

5.3 ROS

5.3.1 Estrogen analogs

In paper III we investigated increases in ROS generation during serum deprivation of PC12 cells. We found that all compounds at 10 µM decreased the immediate deprivation-induced ROS formation, but their ability to reduce ROS levels did not reflect their neuroprotective properties at 10 µM. DHR detects hydrogen peroxide generated through dismutation of superoxide by SOD in addition to peroxynitrite generated from the reaction between superoxide and nitric oxide. There exist conflicting reports on whether 2-ME inhibits SOD or not (Huang et al. 2000, Kachadourian et al. 2001), but 2-ME is reported to decrease DHR-derived green fluorescence, due to inhibition of SOD (Gallina et al. 2016). Our results also show that 2-ME reduces the DHR-derived green fluorescence at both 1 nM and 10 µM which suggests that hydrogen peroxide is involved in the oxidation of DHR in the deprivation-induced ROS generation. The two 2-ME analogs (11f and 13e) also reduced ROS formation at 10 µM. Therefore, this raises the possibility that the two 2-ME analogs reduce the deprivation-induced ROS in the same manner, though not as efficiently as 2-ME when comparing 1 nM concentrations probably due to modification of the steroid.

5.3.2 G36

G36 was included in paper III to explore how an antagonist of GPER would act in deprived PC12 cells. To our surprise, 1 nM of G36 protected against deprivation-induced cell death. In addition, 1 µM also protected against deprivation-induced cell death, whereas both pretreatment and acute treatment with 1 µM G36 protected against glutamate-induced cell death (results not shown). The protective effect of G36 in the deprived PC12 cells was linked to decrease in ROS production in the PC12 cells and in the glutamate treated CGNs. In contrast, research suggests that activation of GPER often has beneficial actions in neurodegenerative diseases revealed from studies with the GPER agonist G1 (Alexander et al. 2017). One plausible explanation, however, for the neuroprotective effects with G36 in our cell models, is inhibition of NO formation through GPER via NOS (Meyer et al. 2014) and superoxide generation via NADPH oxidase (Meyer et al. 2016). These are events that can result in reduced peroxynitrite and hydrogen peroxide formation, resulting in decreased DHR oxidation.

5.3.3 Estradiol molecule

The hydroxyl group in the C3 position on the A ring of the estradiol molecule is important for neuroprotection since modifications to the hydroxyl group result in total loss of neuroprotection (Petrone et al. 2014). In our PC12 cells, 10 µM of 17α-estradiol reduced early ROS production in deprived PC12 cells while 1 nM did not (paper III). Both 17α-estradiol and 17β-estradiol are confirmed to be ROS scavengers at 10 µM, but not as efficient as vitamin C and vitamin E (Fallgren et al. 2007). In contrast to the PC12 cells, acute treatment and the protective pretreatment with 1 nM 17α-estradiol decreased the glutamate-induced ROS production in chicken CGNs (paper IV). Since it is unlikely for the estradiol molecule to directly scavenge ROS at 1 nM (Borras et al. 2010), this suggests a ligand mediated effect occurring in the CGNs by 17α-estradiol. However, there was no ROS scavenging at this concentration in the PC12 cells.

5.3.4 CGNs

Though acute treatment with 1 nM 17α-estradiol reduced the ROS levels and sustained the glutathione levels after glutamate administration, it did not protect against the glutamate-induced cell death (paper IV). Further, the use of DHR as a ROS scavenger caused a small reduction in glutamate-induced cell death, suggesting that ROS is involved only to some

extent in glutamate excitotoxicity in chicken CGNs. In rat CGNs, DHR reduced the glutamate-induced cell death by 60% (Mathisen et al. 2007), and it was confirmed that the toxicity was mediated through peroxynitrite by measuring the levels of 3-nitrotyrosine-modified proteins, a biomarker for RNS (Mathisen et al. 2007). This suggests that peroxynitrite formation is more involved in the glutamate excitotoxicity in rat CGNs than in the chicken CGNs.

5.4 17α-Estradiol and glutamate-induced cell death

In paper IV we demonstrate that 17α-estradiol protects CGNs from glutamate-induced cell death in different time windows with the use of high and low concentrations. Our research group has previously reported that a pretreatment period with both 17α-estradiol and 17β-estradiol at high concentration (10 µM) abolished their neuroprotection from glutamate-induced cell death due to down-regulation of glutathione (Fallgren et al. 2007). Since estradiol mediates neuroprotection in lower concentrations in nanomolar range, we wanted to investigate a low concentration, 1 nM, which is 10 000 times fold lower. Thus, pretreatment with 1 nM protected while acute treatment did not. Acutely, 17α-estradiol is reported to have a dose-dependent neuroprotection against glutamate toxicity in rat cortical neurons, showing protective effects first at 0.1 µM (Yi et al. 2008), consistent with no protection with our results at 1 nM. The protective effects with 17α-estradiol and two other compounds with little ER affinity in this study were shown to be mediated via protein phosphatase preservation and attenuation of the persistent phosphorylation of ERK1/2 associated with neuronal death. Also, these compounds were administrated 24 h before glutamate exposure, and a concentration of 10 nM protected against glutamate toxicity in the same manner, but not at 1 nM. The difference in concentration by pretreatment in this study could be cell type and species dependent, but it raises the possibility that 17α-estradiol protects chicken CGNs through the same mechanism with 1 nM (pretreatment) and 10 µM (acutely treated). As MAPK signaling is well known to be mediated by membrane ERs, it is intriguing to suggest that the protective and non-protective effects of 17α-estradiol may be receptor mediated. However, 17β-estradiol also induces Ca^{2+} influx via L-type Ca^{2+} channels required for its activation of Src/ERK/cyclic-AMP response element binding protein (CREB)/Bcl-2 signaling, proposed to be a potential mechanism for estrogen-induced neuroprotection (Wu et al. 2005). The use of 17α-estradiol in high and low concentrations in our neurons suggests of effects through receptor and/or signaling pathways.

We showed that 17α-estradiol was able to induce minor changes in Ca^{2+} response during glutamate exposure which mirrored the neuroprotection by 17α-estradiol. The difference in Ca^{2+} response between pretreatment with 1 nM and 10 µM was linked to increased NR2B levels with 10 µM. Increase in NMDA receptor subunits indicates increase in total NMDA receptors, thus amplifying Ca^{2+} response during glutamate exposure, and a stronger delayed neuronal disintegration after glutamate exposure (Choi 1992). Also, stimulation of mature extrasynaptic NMDA receptors, which preferably contain NR2B subunits, is associated with pathological outcome during excess glutamate signaling due to their proximity to mitochondria (Bading 2017). Therefore, pretreatment with 17α-estradiol at 10 µM seems to influence NMDA receptor signaling in the wrong direction. The mechanism behind the increased NR2B levels by 17α-estradiol, however, is not clear. Thus, further studies are needed to understand the conditional neuroprotective properties of 17α-estradiol in CGNs.

5.5 Future perspectives

The results in this thesis show that 17α-estradiol has neuroprotective properties, but due to its interference with glutathione regulation, we report that it could have a long-term negative effect on endogenous glutathione levels. Exploring a possible link between p38, MEF2, and AP-1 in the PC12 cells could possibly further reveal the mechanism behind this effect at 10 µM. 17α-Estradiol has much less estrogen activity than 17β-estradiol, but its weak activity should be taken into considerations, especially at higher concentrations. We showed that our PC12 cells lack classical ERs, but the use of G36 strongly suggests that our PC12 cells express GPER. The expression of GPER and possibly other membrane ERs in our cell system should therefore be examined, in addition to receptor binding studies to explore the affinity against these receptors by the compounds. It is shown that a neuronal insult (paraquat) in PC12 cells can modulate the expression of classical ERs in both undifferentiated and neuronal PC12 cells (Gelinas et al. 2004). As transfection with the ERE-luciferase reporter was conducted in non-deprived cell, a possible modulation of classical ERs in deprived cells should also be examined. Also, the protective effect of the 2-ME analogs needs further evaluation due to their effect on cancer cells. Thus, dose-response studies are needed to more precisely assess the concentration-dependent profile of the compounds used in this thesis with regards to their neuroprotective properties in the PC12 cells, and in the CGNs. *In vivo* studies conducted in an ischemic stroke animal model should also be explored to better understand the potential of the compounds.

5.6 Therapeutic considerations

Are effects by estrogens beneficial or harmful? Observations of neuroprotective effects by non-feminizing estrogens are important regarding the Women's Health Initiative (WHI) studies. However, the results from the trial revealed reduced chances of colorectal cancer (Rossouw et al. 2002) which is regarded as a more severe form of cancer than breast cancer. Moreover, a recent study reported that use of HRT was associated with a decreased risk of new-onset atrial fibrillation after myocardial infarction first year after discharge (Bretler et al. 2012). Today, whether estrogen poses beneficial or harmful effects are still debated since postmenopausal women are receiving estrogen against menopausal problems. The making of new non-feminizing compounds is therefore still relevant. As promotion of angiogenesis in the region of ischemia can reduce the volume of cerebral infarction (Meng et al. 2015), caution must be taken with regards to the inhibiting effects on angiogenesis in micromolar concentrations by the two 2-ME analogs (11f and 13e). However, the use of such analogs provides information about the importance of glutathione as a mediator for neuronal survival. Since 17α-estradiol shows protective effects in a concentration which is 10 000 fold lower than its non-protective concentration, the possibility for the use of 17α-estradiol as neuroprotector in the lower nanomolar range should be explored further.

6. Main conclusions

- High concentration (10 μM) of 17α-estradiol does not protect against deprivation-induced cell death in PC12 cells due to down-regulation of endogenous glutathione. The causative factors for the reduced protection were linked to reduction of AP-1 (c-FOS) protein levels and increased protein levels of p-p38.

- 17α-Estradiol protects against deprivation-induced cell death in PC12 cells at 1 nM, a concentration that does not interfere with glutathione levels.

- Modification of 2-ME gives the steroid neuroprotective properties with regards to glutathione regulation in PC12 cells.

- High concentration (10 μM) of 17α-estradiol, but not low (1 nM), protects acutely against glutamate-induced cell death in CGNs, most likely because of lower Ca^{2+} response.

- Low concentration of 17α-Estradiol protects long-term against glutamate-induced cell death, whereas high concentration does not due to up-regulation of NR2B, no reduction in Ca^{2+} response, and decreased glutathione levels.

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