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# The dose-dependent effects of estrogens on ischemic stroke

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The cover depicts a stylized  $17\beta$ -estradiol molecule.

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“Alle Ding’ sind Gift, und nichts ohn’ Gift; allein die Dosis macht, daß ein Ding kein Gift ist”

*(All things are poison and nothing is without poison, only the dose permits something not to be poisonous)*

-Theophrastus Phillippus  
Aureolus Bombastus von  
Hohenheim, a.k.a. Para-  
celsus, 1493-1541

*To my dear family.*

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## List of papers

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Paper 2: Dose-related neuroprotective versus neurodamaging effects of estrogens in rat cerebral ischemia: a systematic analysis. **Ström JO**, Theodorsson A, Theodorsson E. Journal of Cerebral Blood Flow and Metabolism, 2009. 29(8): p. 1359-72.

Paper 3: Different methods for administering  $17\beta$ -estradiol to ovariectomized rats result in opposite effects on ischemic brain damage. **Ström JO**, Theodorsson E, Holm L, Theodorsson A. BMC Neuroscience, 2010. 11: p. 39.

Paper 4: Methods for  $17\beta$ -oestradiol administration to rats. Isaksson IM, Theodorsson A, Theodorsson E, **Ström JO**. Scandinavian Journal of Clinical and Laboratory Investigation, 2011. 71(7): p. 583-92.

Paper 5: Effects of different  $17\beta$ -estradiol doses on cerebral ischemia. **Ström JO**, Ingberg E, Theodorsson E, Theodorsson A. Manuscript.

## Related papers

Mechanisms of Estrogens' Dose-Dependent Neuroprotective and Neurodamaging Effects in Experimental Models of Cerebral Ischemia. **Ström JO**, Theodorsson A, Theodorsson E. International Journal of Molecular Sciences, 2011. 12(3): p. 1533-62.

Incorporated in the Introduction and the Results and Discussion of the thesis.

Hormesis and Female Sex Hormones. **Ström JO**, Theodorsson A, Theodorsson E. Pharmaceuticals, 2011. 4(5): 726-40

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Substantial discrepancies in  $17\beta$ -oestradiol concentrations obtained with three different commercial direct radioimmunoassay kits in rat sera. **Ström JO**, Theodorsson A, Theodorsson E. Scandinavian Journal of Clinical and Laboratory Investigation, 2008. 68(8): p. 806-13.

Methods for long-term  $17\beta$ -estradiol administration to mice. Ingberg E, Theodorsson A, Theodorsson E, **Ström JO**. General and Comparative Endocrinology, 2012. 175(1): p. 188-93.

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Research design and statistical power when publishing "negative findings": comment on "X Chromosome Dosage and the Response to Cerebral Ischemia" Turtzo, et al., 31(37):13255-13259. **Ström JO**, Theodorsson A, Theodorsson E. Letter to the Editor in Journal of Neuroscience, 30 Sep 2011 ([http://www.jneurosci.org/content/31/37/13255.long/reply#jneuro\\_el\\_87193](http://www.jneurosci.org/content/31/37/13255.long/reply#jneuro_el_87193)).

# Abbreviations

ACA	Anterior Cerebral Artery
AMPA	$\alpha$ -Amino-3-hydroxyl-5-Methyl-4-isoxazole-Propionate
ANOVA	Analysis Of Variance
AP-1	Activator protein-1
Apaf-1	Apoptotic protein-activating factor-1
ATP	Adenosine Triphosphate
BA	Basilar Artery
BDNF	Brain-Derived Neurotrophic Factor
BH3	Bcl Homology domain-3
CA1	Cornu Ammonis area-1
cAMP	cyclic Adenosine Monophosphate
CCA	Common Carotid Artery
cGMP	cyclic Guanosine Monophosphate
CV%	Coefficient of Variation in percent
DNA	Deoxyribonucleic Acid
E2	17 $\beta$ -estradiol
ECA	External Carotid Artery
eNOS	endothelial Nitric Oxide Synthase
E/P-ratio	Estrogen group/Placebo group damage ratio in Paper 2
ER	Estrogen Receptor
ERE	Estrogen Response Element
ERK	Extracellular signal-Regulated Kinase
FSH	Follicle Stimulating Hormone
GABA	Gamma-Aminobutyric Acid
GnRH	Gonadotropin-Releasing Hormone
Gr.E/E	Group receiving 17 $\beta$ -Estradiol during entire study in Paper 3
Gr.E/P	Group receiving first 17 $\beta$ -Estradiol and then Placebo in Paper 3
Gr.P/E	Group receiving first Placebo and then 17 $\beta$ -Estradiol in Paper 3
Gr.P/P	Group receiving Placebo during entire study in Paper 3
HERS	Heart and Estrogen/progestin Replacement Study
HRT/HT	Hormone Replacement Therapy/Hormone Therapy
ICA	Internal Carotid Artery
IGF-I	Insulin-like Growth Factor-I
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
IRA	Innovative Research of America
LH	Luteinizing Hormone
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MCA	Middle Cerebral Artery
MCAo	Middle Cerebral Artery occlusion
MRI	Magnetic Resonance Imaging
NADPH	Reduced form of Nicotinamide Adenine Dinucleotide Phosphate

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NFKB	Nuclear Factor Kappa $\beta$
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-Aspartate
nNOS	neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NT-4	Neurotrophin-4
OA	Occipital Artery
PCA	Posterior Cerebral Artery
PI3	Phosphatidylinositol-3
PPA	Pterygopalatine Artery
PUMA	p53-Upregulated Modulator of Apoptosis
RIA	Radioimmunoassay
RIND	Reversible Ischemic Neurologic Deficit
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
SHBG	Sex Hormone-Binding Globulin
SOD	Superoxide Dismutase
STA	Superior Thyroid Artery
TGF	Transforming Growth Factor
Th1	T-helper cell type 1
Th2	T-helper cell type 2
TIA	Transient Ischemic Attack
TNF- $\alpha$	Tumor Necrosis Factor- $\alpha$
TTC	2,3,5-Triphenyltetrazolium hydrochloride
VEGF	Vascular Endothelial Growth Factor
WHI	Women's Health Initiative

# Abstract

Estrogens are a group of female sex hormones that in addition to central roles in reproductive functions also have profound impact on for example brain development, blood vessels, bone tissue, metabolism and the immune system. The dominant endogenous production sites for estrogens in females are the ovaries and adipose tissue, while exogenous sources include combined contraceptive hormone treatments and menopausal hormone therapy. A few decades ago, the observation that females in comparison to men seemed to be protected against cerebral ischemia, and that this benefit was partially lost during menopause, sparked the hypothesis that estrogens protect against stroke. This was later confirmed by epidemiological studies and a large number of experimental animal studies, which motivated extensive clinical trials in which estrogens and/or progestagens were administered with the intent to prevent degenerative conditions rather than to ameliorate menopausal symptoms. However, the results were generally disappointing. The largest study, the Women's Health Initiative (WHI), was discontinued due to the observation of an increased risk of breast cancer, cardiovascular disease and stroke. In parallel, a small number of animal studies in which estrogens were shown to increase damage from cerebral ischemia were published, one of these originating from our laboratory. This was, despite the WHI outcome, a surprising result, since the vast majority of previous animal studies had demonstrated protective effects.

Therefore, in an attempt to explain the discordant results, Paper 1, and later Paper 4, of the current thesis were planned, in which four  $17\beta$ -estradiol administration methods were tested. Substantial differences in serum hormone concentrations resulted from the different methods. Most importantly, the commercially available slow-release pellets used in our earlier experiments resulted in extremely high serum concentrations of  $17\beta$ -estradiol. In Paper 2, 66 published studies that had investigated the effects of estrogens on stroke were meta-analyzed to pin-point the methodological reasons for the result dichotomy. Strikingly, in all six studies in which estrogens had produced damaging effects, the same type of slow-release pellets had been used, although these were used in a minority of the total number of studies. Paper 3 substantially strengthened the hypothesis that administration methods were crucial by showing that repeating the earlier experiment from our laboratory in which pellets had been used, but using a low-dose regimen instead, switched the estrogen effects from neurodamaging to neuroprotective. In Paper 5, an effort was made to challenge the assumption that the dose, and not the administration method per se, was the key factor, however this failed due to large intra-group infarct size variability.

The current thesis adds evidence to the notion that differences in administration methods and their resulting serum concentrations of  $17\beta$ -estradiol constitute a major factor responsible for the dichotomous results in studies investigating estrogens' effects on cerebral ischemia. Even though results from animal stu-

dies are difficult to extrapolate to humans, this has a bearing on the menopausal hormone therapy debate, indicating that the risk of stroke could be reduced if serum concentrations of estrogens are minimized.

## Populärvetenskaplig sammanfattning

Östrogener utgör en grupp av kvinnliga könshormoner med brett spektrum av effekter i kroppen. Förutom de viktiga funktionerna i livmoder och bröst under menstruationscykel och graviditet påverkar östrogener också bland annat hjärnans utveckling, ämnesomsättningen, blodkärlen och immunsystemet. Östrogener produceras i störst mängd i äggstockarna och fettvävnaden, men intas också i form av kombinerade p-piller och hormonterapi för mildrande av klimakteriesymtom. För ett par årtionden sedan noterade forskare att kvinnor som efter klimakteriet intog östrogener verkade ha en minskad risk för cerebral ischemi, eller "stroke"; en dödlig sjukdom som orsakas av att ett kärl i hjärnan täpps till av en blodpropp. Under senare delen av 1990-talet bekräftade man denna observation genom att visa att råttor som fick östrogener och sedan åsamkades stroke klarade sig bättre än de djur som istället fick placebobehandling innan stroke. Man startade därför flera stora studier med syftet att undersöka om östrogen kunde ges med syfte att undvika olika sjukdomar, t.ex. cerebral ischemi, istället för att bara lindra klimakteriesymtom. I den största av dessa studier (kallad Women's Health Initiative), var resultaten en besvikelse, och hela försöket avbröts i förtid på grund av ökad risk för bröstcancer, kranskärlssjukdom och stroke i gruppen som fått könshormonerna. Parallellt med dessa publicerades ett litet antal djurexperimentella studier som också pekade på att östrogener ökade skadan av cerebral ischemi. Det fanns alltså en diametral motsättning i resultat både bland människostudier och bland djurstudier.

En av djurstudierna som visade att östrogener var skadliga kom från vårt laboratorium. För att undersöka orsakerna till de oväntade resultaten planerades den studie som blev Paper 1, och senare Paper 4, i nuvarande avhandling, där fyra olika metoder att ge östrogener till råttor undersöktes. Resultaten visade betydande skillnader i serumkoncentrationerna av östrogener mellan metoderna, och att den subkutana pellet som bl.a. använts i vårt laboratorium gav synnerligen höga koncentrationer. I Paper 2 analyserades data från 66 tidigare publicerade djurexperimentella artiklar där östrogeners effekt på stroke undersöktes. Denna metaanalys visade att i samtliga djurstudier där östrogener rapporterats skadliga vid stroke hade hormonet tillförts via subkutana pellets. Paper 3 visade att om det tidigare strokeexperimentet på djur, där östrogenerna medfört ökad hjärnskada gjordes om till punkt och pricka, förutom att de subkutana pelletarna byttes ut mot en lågdosmetod, fick hormonet istället en skyddande effekt på hjärnan. I Paper 5 undersöktes om dosen i administrationsmetoden var den avgörande faktorn, något som ej kunde påvisas i aktuell studie, sannolikt på grund av oväntat stor slumpmässig variation i utbredningen av hjärninfarkterna.

Avhandlingen antyder således att valet av metod att tillföra östrogener och de resulterande östrogenkoncentrationerna i serum är orsaken till de spretiga resultaten vad gäller effekten av östrogen på cerebral ischemi i råttmodeller. Detta har viss bäring på debatten om hormonterapi i klimakteriet, även om överföring

av djurbaserade data till människor är svårt. Men kanske kan man reducera risken för stroke och samtidigt behålla hormonersättningens fördelar genom att minska den intagna östrogendosen.

# 1. Introduction

In 2005, Theodorsson and Theodorsson found that  $17\beta$ -estradiol increased ischemic lesions in a rat model of stroke [1], contradicting a large majority of studies indicating the opposite. At the time, this was a highly surprising finding, sparking an interest in investigating whether underlying methodological factors could contribute to the dichotomy. This has now been an ongoing quest for more than five years, and constitutes the cornerstone of the current thesis.

Since the effect of estrogens on ischemic stroke is the subject of the thesis, a description of cerebral ischemia (1.1), followed by a presentation of the female steroid hormone family of estrogens (1.2) forms the backbone of the Introduction. This is followed by a review of the previous knowledge about estrogens' effects on stroke (1.3). Another important concept in the current thesis is "hormesis", reflecting the phenomenon that a substance can exert diametrically different effects on an endpoint depending on dose, an issue dealt with in the last section of the introduction (1.4).

## 1.1 Focal cerebral ischemia

Focal cerebral ischemia is commonly referred to as "stroke", a term which also includes intracranial hemorrhage. Stroke is one of the leading causes of death in the industrialized world, and also results in a considerable burden of long-term morbidity for the society [2]. Focal cerebral ischemia constitutes approximately 80-90 % of strokes [3], and therefore massive resources have been allocated in the attempt of finding treatments for this devastating disease. But even though more than a thousand treatments have been proven effective in animal studies, only one – thrombolysis – has been shown to be of clinical value [4]. In parallel with the continued search for effective treatments, research to find preventive measures to avoid focal cerebral ischemia remain crucial. Anti-smoking campaigns and antihypertensive and blood lipid lowering treatments have reduced the general burden of atherosclerosis – a major causative factor in stroke, but central questions remain to be answered. Significantly, the question still remains open whether it is beneficial or detrimental to replace sex hormones in postmenopausal women.

The term "focal cerebral ischemia" or "ischemic stroke" describes the event that a cerebral vessel is occluded, causing a part of the brain to suffer from ischemic damage due to reduced or total loss of blood flow. In the clinical situation, the occlusion is caused by an embolus or a thrombus. While thrombi are direct consequences of local atherosclerosis, emboli most often originate from the heart or the carotid arteries [5]. When the blood flow to a part of the brain is interrupted, the functional loss is instant, and the pathophysiological process rapidly progresses to irreversible damage and massive cell death. In the clinical as well as in the animal experimental situation, the symptoms are mainly direct consequences of the loss of function in the affected brain region. For example, if the

most commonly affected vessel, the middle cerebral artery (MCA), is occluded on the left side, a large part of the functions of the parietal, temporal and frontal lobes, including speech and most of the contralateral motor and sensory capacity, will be lost.

The pathophysiological process of focal cerebral ischemia is complex and involves several components, including excitotoxicity, edema, oxidative stress, apoptosis, necrosis and inflammation [6-10]. The reduction of blood flow to the brain area rapidly causes a shortage of glucose and oxygen for the energy-demanding neurons, hampering the mitochondrial adenosine triphosphate (ATP) production. This ATP deficit in turn deactivates the entire cell machinery, including the crucial ion pumps. The loss of function in the ion pumps makes the cell swell, causing an intracellular edema that can be severe enough to cause brain herniation and sudden death. In addition, as the ion pumps stop working, the cell becomes depolarized, leading to instant loss of function and an inflow of calcium. The elevated calcium concentrations activate various detrimental calcium dependent enzymes, in turn damaging the cell by degrading cytoskeletal proteins and deoxyribonucleic acid (DNA), and by increasing the generation of free radicals [8, 10]. Moreover, the depolarization causes glutamatergic neurons to release glutamate into the synapses, stimulating N-methyl-D-aspartate (NMDA) and  $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) glutamate receptors, contributing to increased influx of  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  ions and further depolarization in a vicious cycle called "excitotoxicity". In the process, the energy deficient mitochondria lose their integrity, leading to production of free radicals and leakage of pro-apoptotic enzymes in the cytosol, thus activating the caspase system [6, 7]. From this havoc of ceased cell functions, the cell can die by either necrosis or apoptosis. The necrotic debris triggers an acute and prolonged inflammatory process in the brain, characterized by activation of microglia, production of inflammatory cytokines and infiltration of various inflammatory cells, including neutrophils, T-cells and monocytes/macrophages, into the damaged tissue. The inflammation also contributes to the tissue damage, and especially the early inflammatory cell infiltration and cytokine production seem to be predominantly deleterious [9]. Thus the development of the infarct is a process that, even though rapidly progressing to irreversibility for some cells, proceeds for several days [8](Figure 1).

When describing the pathophysiology of stroke, it is vital to emphasize the heterogeneity in the affected brain volume. Even though the main supplying vessel may be totally occluded, collateral circulation will produce a gradient of blood flow, from almost no blood flow in the infarct core, via moderately reduced blood flow, to normal blood flow just outside the affected volume. The relatively large volume of brain tissue in which the reduction in blood flow is severe enough to cause loss of function, but not severe enough to cause the almost instant cell death is usually referred to as the "penumbra". After a few days, when the infarct has matured, a part of the penumbra will have been restored while the rest is

infarcted. It is the penumbra that most therapies, including revascularization therapy, aim to save.

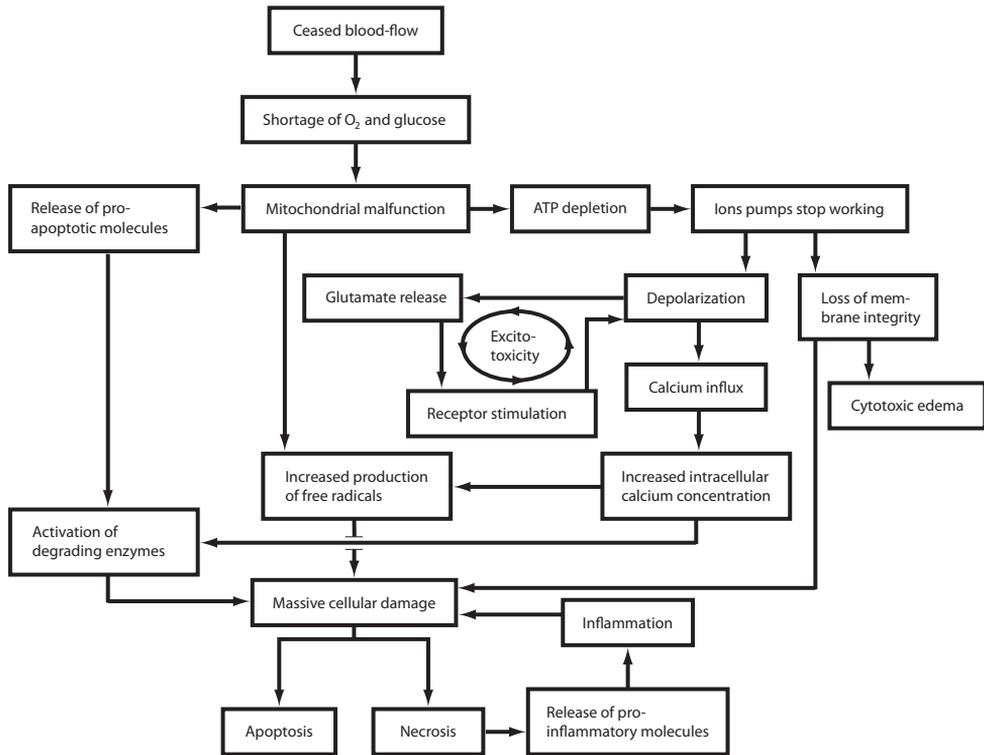


Figure 1. Overview of pathophysiological mechanisms in focal cerebral ischemia. The ceased blood flow leads to a rapidly progressing mitochondrial malfunction that causes generation of free radicals, release of pro-apoptotic molecules and depletion of ATP, in turn hampering the entire cellular machinery. Excitotoxicity, shown in the middle of the figure, is a self-perpetuating cycle initiated by the ion pump cessation and subsequent depolarization. All events converge in massive cellular damage that leads to cell death in the form of apoptosis or necrosis, in turn triggering an inflammatory reaction that further damages the tissue.

## 1.2 Estrogens

Estrogens are a family of steroid hormones that are produced endogenously or prescribed for amelioration of peri-menopausal symptoms and for contraceptive purposes. The endogenous production of the hormones takes place in the gonads, and to a smaller extent in the adipose tissue stroma, by aromatization of a structure called the A-ring (which is crucial for the high-affinity binding to the nuclear estrogen receptors; ER) and elimination of the 19<sup>th</sup> carbon from androstenedione or testosterone [11]. Estrogens are also produced in smaller amounts in other tissues expressing aromatase, including liver, bone tissue and the brain [12], where it is thought to mainly have local effects [13, 14]. In the ovaries, the estrogen production is cyclical. During the follicular phase (“pro-estrus” in rodents), estrogens are produced in increasing amounts by the gra-

nulose cells surrounding the aspiring gametes in competing follicles, stimulated by gonadotropin-releasing hormone (GnRH) pulses from the hypothalamus via follicle stimulating hormone (FSH) from the pituitary gland. When the gradually increasing production of estrogens inhibits FSH secretion, only the largest and most FSH-sensitive follicle survives. During mid-cycle, the estrogens, possibly in concert with progesterone, suddenly stop inhibiting the gonadotropins, and on the contrary trigger a surge in FSH and luteinizing hormone (LH), by which ovulation is initiated. During ovulation, the cells that surrounded the ovum are transformed into a luteal body that starts to produce progesterone in addition to its ongoing estrogen production. The estrogen/progesterone production is at its maximum about one week after ovulation, which in humans is the time for nidation. If no conception takes place, the luteal body degenerates and the estrogen and progesterone levels decrease, allowing increased levels of FSH that stimulates new competing follicles in the next cycle [13, 15]. In female rats, the cycle phases are called diestrus, proestrus and estrus, where the preovulatory estrogen build-up occurs in proestrus, and the ovulation during estrus [16]. An important difference in comparison to humans is that once the fertile period has passed and new follicles are developed, the endometrium is not expelled, but reabsorbed. Further, the rat estrous cycle is only 3-5 days long, in contrast to the 28-day average in women [17].

The most potent naturally occurring estrogen – 17 $\beta$ -estradiol – mainly circulates in the blood bound to sex hormone-binding globulin (SHBG) and albumin; however it is the small unbound fraction that is biologically active. An equilibrium exists between the bound and free fraction, which for example makes increased levels of SHBG decrease the amount of biologically active hormone [13].

Concerning signal pathways, the nuclear ER $\alpha$  and ER $\beta$  have classically been credited with most of the estrogens' biologic effects [18]. ER $\alpha$  is expressed in uterus, vagina, ovaries, mammary gland, endothelial cells and vascular smooth muscle, whereas ER $\beta$  is most highly expressed in prostate and ovaries, with lower degrees of expression in lung, bone and vasculature [13]. In tissues where both ER $\alpha$  and ER $\beta$  are expressed, ER $\alpha$  is often pro-proliferative, while ER $\beta$  counteracts proliferation [19]. The expression of the two receptor types in the brain also differs, with ER $\alpha$  widely spread in regions such as hippocampus, amygdala, hypothalamus, and brainstem, whereas ER $\beta$  is mainly expressed in the hippocampus and selected hypothalamic nuclei [12]. However, the pathways for estrogens' effects are even more multifaceted and complex. The classical pathway is complemented by actions mediated by membrane bound receptors, such as G-protein coupled receptor-30 [20], and by direct effects including redox cycling [21]. The nuclear receptors can interact with other transcription factors to affect genes that lack an estrogen response element (ERE) [22, 23], and ER $\alpha$  and ER $\beta$  have also been found to reside in the cellular membrane, possibly contributing to the rapid effects of estrogens [24]. Further, at very high doses, 17 $\beta$ -estradiol is known to cause down-regulation of its own receptors [25] at the same time as

stimulating other receptors of the nuclear receptor superfamily, thus activating a totally different set of genes in the supraphysiological compared to the physiological concentration range [26].

During the human menstrual cycle, the fluctuating estrogen and progesterone concentrations cause the endometrium to first proliferate, then to differentiate and finally to go into apoptosis or be expelled, and the breasts to vary in size. Analogous effects are found in rodents. Furthermore, estrogens are involved in the pubertal development in girls, causing growth of the vagina, uterus and breasts. However, even though most renowned for their feminizing properties, estrogens exert a wide range of effects in the body. Examples include growth of axillary and pubic hair, genital and nipple pigmentation during pregnancy, increased bone mineralization, modulation of lipoprotein patterns, effects on the clotting cascade, altered immune response and multiple actions in the brain on mood, memory, neurodevelopmental and neurodegenerative processes [12, 13].

The first pass metabolism of orally administered estrogens is high, and  $17\beta$ -estradiol is rapidly converted to less active forms, such as estrone and estriol, in the liver [13, 27]. Other degradation products originating from the liver, that also may possess estrogenic effects, include sulfate conjugates, glucuronide conjugates and hydroxyestradiols [27]. The conjugated estrogen variants are both excreted in the bile and urine; however estriol is dominant among the estrogens excreted in the urine. Estrogens also undergo enterohepatic recirculation since the conjugated forms that are secreted in the bile are hydrolyzed by bacterial enzymes to subsequently be reabsorbed. The enterohepatic circulation is important when considering estrogen pharmacokinetics and is, to give clinical examples, the reason why antibiotics and dietary fibers may alter the effect of contraceptives [13].

After menopause, serum concentrations of estrogens in women decrease to levels close to or below those in males. In women, the menopause is initiated by ovulatory failure, which in turn depends on loss of ovarian reserve with successively decreasing number and quality of pre-ovulatory follicles. This process takes place over several years, often with gradually disappearing cyclicity, ultimately resulting in low levels of estrogens as well as progesterone. It should be noted, however, that when menopause (i.e. the last menstrual bleeding) occurs there is still a small reserve of follicles which may cause varying estrogen production for about another five years. In female rats, however, the natural process of reproductive senescence is not driven by decreased ovarian reserve, but rather by increasingly abnormal gonadotropin release patterns, and thus seems to be determined more by the hypothalamus. At an age of 6-10 months in Sprague Dawley rats, this leads to a months-long non-cyclical period called "persistent estrus" with sexual receptivity, vaginal cornification, elevated levels of estrogens and low levels of progesterone, followed by a permanent anestrus with low levels of both estrogens and progesterone. Hence, even though the final stage in

women and female rats is similar, the persistent estrus of rats is unparalleled in humans [28, 29]. This underlines the difficulties in trying to model menopause in animals, and in extrapolating such data to human populations.

### **1.3 The effects of estrogens on stroke**

In the 1990's, several studies suggested neuroprotective effects of female sex or estrogens in animal models of cerebral ischemia [30-32]. This supported a hypothesis of estrogen neuroprotection that earlier had been postulated from the clinical observation that women are less likely than men to suffer from stroke, and that this protection diminishes by the advent of menopause [33]. Several previous epidemiological studies had corroborated this hypothesis by indicating a decreased incidence of stroke incidence in women on hormone replacement therapy (HRT) compared to women not using HRT [34-37]. Encouraged by the potential of estrogens as a mean of preventing illnesses, including stroke and other cardiovascular diseases, substantial research efforts were invested in further studies of the matter. However, later findings have been contradictory regarding the effects of estrogens on stroke, exemplified by the large randomized placebo-controlled trial WHI, which was discontinued due to the observation of increased incidences of breast cancer, stroke and cardiovascular disease, thus apparently antagonizing the hypothesis that estrogens are neuroprotective [38].

Interestingly enough, a similar dichotomy exists in the animal experiment literature. Despite the fact that the aforementioned initial studies demonstrated protective effects of estrogens, confirmed in a large number of later publications (for example [39, 40]), there are examples of studies in which estrogens have indeed augmented cerebral ischemia [1, 41-45]. However, it should be emphasized that the studies reporting protection are in massive majority, constituting about 90 % of the literature concerning rats.

Several explanations have been put forth to explain the diametrically different results of estrogens on cerebral ischemia. Some of these have focused on the difference between the randomized trials on one hand and the epidemiological studies and majority of animal studies on the other hand, for example proposing the "window of opportunity" hypothesis (also called the "timing hypothesis"). The window of opportunity hypothesis, probably the theory with the largest number of followers, states that after menopause (or surgically induced estrogen deprivation) estrogens must be administered within short to have beneficial effects. In the WHI, the women were on average 61 years old, thus having had low estrogens for almost a decade before initiating estrogen therapy. The experimental evidence for this hypothesis is however relatively weak, and mainly relies on a couple of studies in which protection was observed after immediate administration, but not after a longer period of washout [46]. Contradicting the window of opportunity hypothesis, subgroup analyses from the WHI did not indicate that age of initiation influenced the effects of hormone therapy on stroke

risk [47-49]. A variant of this suggestion is that the difference in age of the experimental animals in comparison to the women in the studies could be the explanation, and that younger individuals are more likely to benefit from estrogen treatment [50, 51].

It has also been suggested, mainly with reference to the differences between the randomized trials and the animal experiments, that differences in type of the administered estrogen may be the explanation. Conjugated equine estrogens have been administered in most human studies performed in the USA, notably in the WHI, while animal researchers commonly use 17 $\beta$ -estradiol. Though, the conjugated equine estrogen substance premarin has also been administered in several rat studies, with consistently protective results, and in most of epidemiological studies in which protective effects were seen, mainly conjugated estrogens were consumed [34-37], casting this hypothesis in doubt.

Other hypotheses purporting to explain the differences in animal studies range from choice of strain and prevalence of concurrent diseases [52] to discrepancies in middle cerebral artery occlusion (MCAo) surgery techniques. Dose as a possible contributing factor has also previously been suggested, however without further analysis [50]. Hence, in summary, the suggestions for the explanation of the discrepant results have focused on a number of methodological issues, although detailed investigations into the different issues have insofar been scarce.

### *1.3.1 Mechanisms of estrogens' effects on cerebral ischemia*

Numerous explanations concerning mechanisms of estrogens effects in cerebral ischemia have been put forth, mostly focusing on estrogens' protective properties, but also with some suggestions of detrimental pathways. The investigation of possible mechanisms is important not only to enable utilization of the hormone's beneficial effects, but can also provide clues to the nature of the dichotomous results mentioned above (section 1.3). A detailed account of the mechanisms that have been suggested is therefore called for as a mean of further shedding light on the evidence at hand. The following sections will review the five most extensively investigated potential neuroprotective mechanisms, namely decreased oxidative stress (section 1.3.1.1) decreased inflammation (section 1.3.1.2), decreased apoptosis (section 1.3.1.4), growth factor regulation (section 1.3.1.5) and vascular modulation (section 1.3.1.6), and the three suggested neurodamaging mechanisms increased oxidative stress (section 1.3.1.1), increased inflammation (section 1.3.1.2) and increased excitotoxicity (section 1.3.1.3). Each section consists of a brief summary of data supporting the mechanism hypothesis, when required including a complementary description of pathways (Figure 2).

Although not reviewed below, as research efforts into their pathways are still in the early stages, a number of additional suggested protective mechanisms also deserve mentioning. These include increased recruitment of stem cells from the subventricular zone [53], avoidance of apoptosis by balancing phosphatase acti-

vity [21] and decrease of excitotoxicity by reducing NMDA-signaling [54, 55] (it should be noted that the opposite – that estrogens may increase excitotoxicity and thereby increase ischemic damage – is reviewed in section 1.3.1.3).

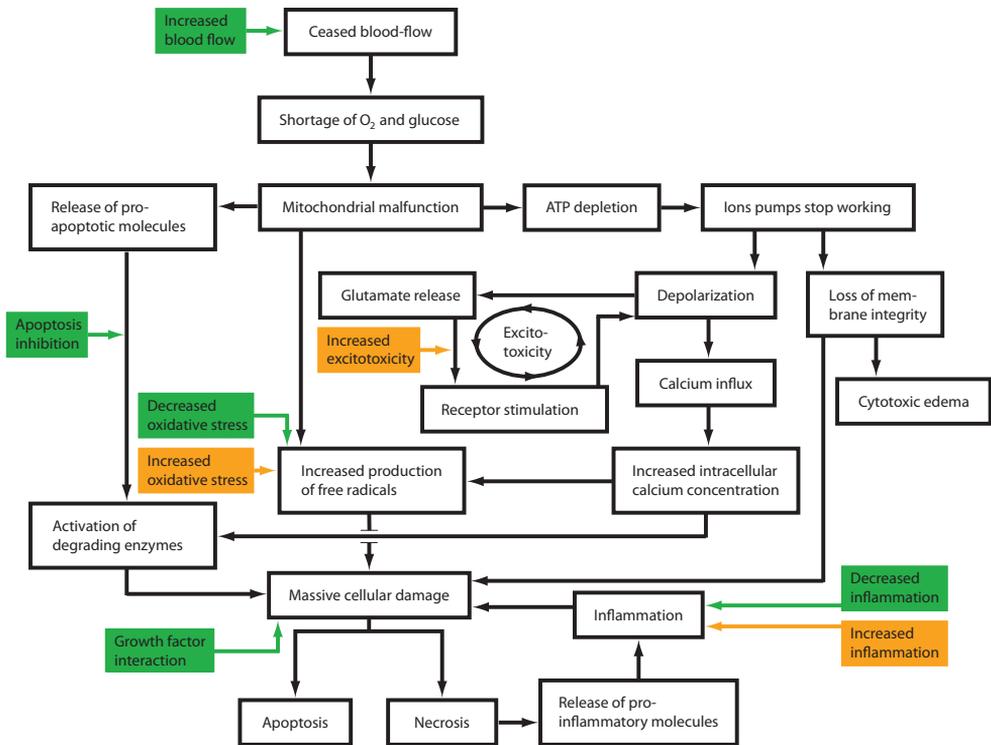


Figure 2. The stroke-pathophysiology figure above (Figure 1 in section 1.1) is here complemented with the most common suggestions for estrogens' protective (green boxes) and detrimental (orange boxes) effects in cerebral ischemia. Increased blood flow, inhibition of apoptosis, decreased oxidative stress, decreased inflammation and interaction with growth factors have all been claimed to be protective estrogenic mechanisms, while increased excitotoxicity, increased inflammation and increased oxidative stress have been proposed as the damaging counterparts.

### 1.3.1.1 Decreased and increased oxidative stress as mechanisms of estrogenic neuroprotection and neurodamage

As mentioned above (section 1.1), oxidative stress is an important mechanism in cellular damage in general and cerebral ischemia in particular. Ischemia prompts mitochondria to produce reactive oxygen species (ROS), which cause direct damaging oxidative reactions such as lipid peroxidation, as well as triggering apoptotic cascades. The cell carries intricate defense systems against oxidative damage, including scavenging activity by superoxide dismutase (SOD), glutathione peroxidase, and catalase, and further detoxification by small molecules such as glutathione, ascorbic acid, and  $\alpha$ -tocopherol. However, during cerebral ischemia, especially reperfusion, these systems are generally overrun by the massive oxidative stress [10]. Estrogens have been stipulated to exert their neuroprotective effects both through direct chemical effects and indirectly via

upregulation of the cell's anti-oxidative defense mechanisms (Figure 2)[21].

Direct anti-oxidative effects have been found in several studies. More specifically, estrogens have been reported to prevent intracellular peroxide accumulation in an ER-independent manner [56], to decrease ROS production [57], to limit lipid peroxidation [58-61], to protect against  $\text{FeSO}_4$ -mediated oxidative stress [62], and to decrease hydrogen peroxide concentrations [63]. In one of these studies, no extra protection was afforded by adding known potent free radical scavengers, indicating that estrogens exert all the protective effects available through anti-oxidative mechanisms [59]. Further,  $17\alpha$ -estradiol, a less feminizing enantiomer of  $17\beta$ -estradiol, has been shown to protect against glutamate and hydrogen peroxide stress to a similar extent as  $17\beta$ -estradiol, indicating the importance of receptor-independent pathways [64, 65]. Anti-oxidative mechanisms have also been suggested merely on the basis that estrogens can protect against oxidative stress [66, 67], although it should be emphasized that the protection against an oxidative assault is not necessarily dependent on a primarily anti-oxidative mechanism. A further mechanism for estrogens' direct anti-oxidative effect was proposed by Prokai et al., providing evidence that estrogens can engage in a redox cycle in which estrogens turn into a quinol when eliminating a radical, subsequently to be converted back to the parent estrogen using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a reducing agent [68, 69]. However, the direct anti-oxidative effect still needs to be demonstrated in relevant biological settings in whole-animal models, and this mechanism is especially complex to assess because of the difficulties in separating genomic from non-genomic actions.

Indirect anti-oxidative effects of estrogens have also been reported, including attenuation of microglial superoxide release [70], increase of glutathione reductase, gamma-glutamylcystein synthetase, glutaredoxin and glutathione [71-75], increased MnSOD activity [76, 77] and expression [78, 79], upregulation of Cu/ZnSOD expression [78], reduction of free radical production via an increase mitochondrial efficiency [80, 81], attenuation of NADPH oxidase activation [82, 83] and the decrease of the oxidative stress marker nitrotyrosine [78]. These effects have been found to result at least in part from nuclear ER-mediated upregulation of anti-oxidative proteins [21].

Estrogens have also paradoxically been shown to increase oxidative stress, and thereby possibly augment ischemic damage (Figure 2)[43, 84]. The reported pro-oxidative effects include increased mitochondrial ROS production [85, 86], oxidative DNA-damage in sperm and ovarian surface epithelium [87, 88], reduced levels of anti-oxidant proteins in rat brain [89], promotion of oxidative damage in rat liver cells [90] and increased ROS-production from the estrogen metabolites 2-methoxyestradiol and 4-hydroxyestradiol [91-93]. However, these pro-oxidative effects of estrogens have mainly been reported from in vitro experiments and in other tissues than the brain, while studies on the nervous system almost uniformly have found estrogens to exert anti-oxidant properties [84]. This could

possibly reflect tissue-specific estrogen response patterns, which has been proposed to result from differences in cellular balance of ER- $\beta$  versus ER- $\alpha$  [84]. In other words, a pro-oxidative mechanism for estrogens' detrimental effects in stroke has relatively little experimental evidence.

#### 1.3.1.2 Anti- and pro-inflammatory actions as mechanisms of estrogenic neuroprotection and neurodamage

As mentioned above (section 1.1), the inflammatory process is considered an important component of the pathophysiology of stroke. Experiments in rats have shown that intraventricular administration of tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 exacerbates stroke damage, suggesting a detrimental role of inflammation in the ischemic process [94-96]. Further support for the importance of inflammation in the pathophysiological process is found in the observation that blockage of pro-inflammatory cytokines ameliorates ischemic damage [96-101].

The anti-inflammatory properties of estrogens have been demonstrated in a large number of studies, and are commonly taken as important mechanisms for estrogens' neuroprotective effects in stroke [96]. Estrogens have been shown to induce a wide range of anti-inflammatory effects via for example reducing leukocyte adhesion [102-104], decreasing pro-inflammatory cytokine production [46, 51, 105-110], decreasing monocyte activation [111] and altering the microglial activation pattern [112]. Both leukocytes and microglia express ER, offering a pathway for estrogens' actions in inflammatory processes [96, 111, 113], and ER activation is for example thought to regulate inducible nitric oxide synthase (iNOS) transcription [114]. The classical pro-inflammatory cytokines IL-1, IL-6 and TNF- $\alpha$  lack ERE, but are thought to be affected by for example activated ER's down regulation of nuclear c-Jun and JunD, leading to decreased occupation of activator protein-1 (AP-1) which in turn could alter the expression of TNF- $\alpha$  [96, 115].

However, the abovementioned studies designed to investigate estrogens' actions in inflammation have to a large extent been performed in cell cultures, under conditions difficult to extrapolate to the situation in intact organisms. Of the studies performed in animals, most have focused on other organs than the brain. This could potentially lead to misinterpretation if the data are extrapolated to estrogens effects in cerebral ischemia. The effects of estrogens on inflammation are in many respects organ specific, vividly exemplified by the estrogen-induced prostatitis in rats [116] in contrast to the amelioration of soft tissue inflammatory conditions [117]. In order to benefit from quality and precision rather than quantity and potential imprecision, we therefore narrow our focus to studies performed with the intention to assess effect on cerebral inflammation. These are comparatively few, but include experiments having shown estrogens to limit the activity of the pro-inflammatory transcription factor nuclear factor kappa B (NFkB) in a rat MCAo model [118], decrease leukocyte adhesion both before and after transient forebrain ischemia in rats [102], reduce number of microglia

and astrocytes in mice [119], decrease cytokine production in animal models of MCAo [105] and NMDA-induced toxicity [51], block cyclooxygenase-2 activity and prostaglandin E2 production after IL-1 $\beta$  administration in rats [110], reduce iNOS activity [114], and decrease monocyte activation and recruitment in response to lipopolysaccharide (LPS) [111]. In two studies, the importance of anti-inflammation for estrogens' actions have been demonstrated by the lack of 17 $\beta$ -estradiol neuroprotection in iNOS knockout mice [120] and mice treated with the iNOS inhibitor aminoguanidine [121].

A second paradox concerning estrogens effects on ischemia, paralleling the aforementioned anti- or prooxidative effects (section 1.3.1.1), is that pro-inflammation is one of the suggested mechanisms for estrogens' ability to increase damage in cerebral ischemia [45, 122]. In several rat experiments, estrogens have been reported to potentiate leukocyte adhesion, increase P-selectin and myeloperoxidase enzyme activity in cerebral ischemia [45, 123], increase TNF- $\alpha$ , toll-like receptor-2 and IL-12 in response to LPS stress [124, 125], increase IL-1 $\beta$  in a NMDA-toxicity model [51] and to worsen functional outcome in a model of chronic cerebral inflammation [126]. The possible implications for this paradox are further discussed in the Results and Discussion section.

### 1.3.1.3 Increased excitotoxicity as a mechanism of estrogenic neurodamage

Excitotoxicity is a well-established feature of cerebral ischemia, and contributes to the pathophysiology by a series of events characterized by abnormal excitation of neurons due to pathological release of excitatory neurotransmitters from damaged cells.

It has been stipulated that estrogens could augment the pathological process in cerebral ischemia by potentiating the excitotoxicity since estrogens have been reported to increase NMDA mRNA in the cerebral cortex [127], increase NMDA-binding sites in the hippocampal cornu ammonis area-1 (CA1) region [128, 129], increase dendritic spine density or decreased ovariectomy-induced dendritic spine loss in CA1 [128, 130-132], increase the sensitivity of CA1 pyramidal cells to NMDA receptor-mediated synaptic input [128], facilitate seizure activity [133], augment long term potentiation [134, 135], increase the excitability of different neurons [136, 137], decrease glutamate-uptake by astrocytes [138] and to facilitate kainate-induced currents via cyclic adenosine monophosphate (cAMP)-dependent phosphorylation [139]. It is likely that a substance that facilitates NMDA activity and increases excitability could potentiate excitotoxicity and augment ischemic damage. In line with this hypothesis, it has been purported in several articles that decreased excitotoxicity, either by reducing the number of collaterals [140-142] or potentiating gamma-aminobutyric acid (GABA)-ergic transmission [143, 144], is associated with amelioration of ischemic damage. However, as a whole the direct evidence for increased excitotoxicity as a mechanism for estrogens' possible damaging effects in cerebral ischemia is scarce, and the hypothesis must be considered relatively weak.

#### 1.3.1.4 Decreased apoptosis as a mechanism of estrogenic neuroprotection

Apoptosis is, as aforementioned (section 1.1), a major mode of cell death in ischemic brain injury [6, 7]. Ischemia triggers mitochondria to produce ROS, which do not only directly damage lipids, proteins and nucleic acids in the cell, but also activate various intracellular pathways that return to the mitochondria to induce apoptotic cell death, in part through regulation of pro- and antiapoptotic proteins such as the Bcl-2 family [6]. The Bcl-2 family is an essential group of proteins that regulate the integrity of the mitochondrial membrane. It is divided into three subgroups based on structural homology: antiapoptotic proteins including Bcl-2, Bcl-XL and Bcl-w; proapoptotic proteins such as Bax and Bak and the Bcl homology domain-3 (BH3)-only proteins including Bad, Bim, Noxa and p53-upregulated modulator of apoptosis (PUMA) [6]. An overweight of proapoptotic proteins at the membrane triggers the release of cytochrome c into the cytosol, which in turn combines with apoptotic protein-activating factor-1 (Apaf-1) and procaspase-9 to activate various caspases, such as caspase-3. The caspases are proteins performing the cellular degradation in apoptosis, exemplified by caspase-3's cleavage of DNA repair enzymes leading to DNA damage [145]. Another feature of apoptotic cell death is the seemingly mandatory increase in expression of the so-called immediate early genes, such as c-Jun and c-Fos [146, 147], which can be used as markers of apoptosis [148]. The importance of apoptosis in cerebral ischemia is suggested by the neuroprotection afforded by increased expression of the antiapoptotic Bcl-2 [149, 150] and by the ischemia-induced upregulation of proapoptotic proteins in animal models of cerebral ischemia [151].

In a number of studies, estrogens have been reported to reduce apoptosis. The antiapoptotic effects of estrogens include blocking the ischemia-induced reduction of Bcl-2 following MCAo [150, 152], reducing caspase-3 after global ischemia [153], increasing expression of Bcl-2, Bcl-w and Bcl-XL while decreasing Bax, Bad and Bim [154-160], attenuating injury-mediated DNA fragmentation [161], reducing the level of the 120 kDa caspase-mediated spectrin breakdown product [161], decreasing c-Fos induction [148], limiting apoptosis induced by staurosporine in cell cultures [162], inducing cyclic guanosine monophosphate (cGMP)-dependent expression of thioredoxin – a redox protein with potent antioxidative and antiapoptotic properties [163] – as well as preventing glutamate-induced translocation of cytochrome c from mitochondria to cytosol [164]. ER activation is also thought to limit apoptosis through increased expression of components in oxidative phosphorylation, making energy production more stable and thus maintaining mitochondrial membrane integrity [21].

#### 1.3.1.5 Growth factor regulation as a mechanism of estrogenic neuroprotection

Estrogens are known to regulate growth factors, an attribute that has been suggested as another mechanism for the hormones' beneficial effects in cerebral ischemia [165, 166]. Growth factors contribute to improved outcome after ischemic stroke by facilitating recovery as well as decreasing apoptosis, thereby

reducing infarct size [167]. This mechanism considerably overlaps with apoptosis, even if the extensive research focused on estrogens interaction with growth factors merits special attention. Also, the positive, possibly neuroprotective, effects of estrogens on neural cell proliferation, synaptogenesis, modulation of synaptic connectivity and regeneration [168, 169] are probably mediated through regulation of growth factors and neurotrophins, including transforming growth factor (TGF)- $\beta$ , insulin-like growth factor (IGF)-I, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) [170-175].

17 $\beta$ -estradiol regulates the transcription of numerous growth factor genes through ERs' binding to ERE in gene promoters. The factors influenced in this manner include for example vascular endothelial growth factor (VEGF) [176], TGF- $\alpha$  [177], tau [178], BDNF, NT-4 and NGF [165, 172]. ERs not only co-localizes with and regulates the expression of neurotrophins and their cognate receptors, but estrogens and neurotrophins also share converging signaling pathways in the mitogen-activated protein kinase (MAPK) cascade, which includes activation of B-Raf and extracellular signal-regulated kinases (ERK), in turn regulating a broad array of cytoskeletal and growth-associated genes [179]. Additional evidence implying that estrogens exert their positive effects via growth factor interaction includes the cooperation with IGF-I to exert neuroprotection, possibly by sharing the MAPK and phosphatidylinositol-3 (PI3)/Akt signaling pathways [170, 180]. Interestingly, IGF-I receptor blockade prevents estrogen neuroprotection while the ER antagonist ICI 182,780 can block IGF-I neuroprotection [181, 182]. Similar results have been seen in models of cerebral ischemia [165, 183]. In another study, a combination of IGF-I and 17 $\beta$ -estradiol did not add any extra protection against ischemia compared to the two substances administered separately [184], emphasizing the relation between estrogens and growth factors as a protective mechanism in stroke. Moreover, estrogens have been postulated to promote recovery after stroke by directly regulating genes required for growth, such as tau microtubule-associated protein [178], growth-associated protein-43, [185], structural lipoproteins such as apolipoprotein E [186], and neurofilament proteins [187]. Thus, ample evidence exists for the notion that estrogens' increase in the activity of growth factors is a major mechanism of the hormones' neuroprotection.

### 1.3.1.6 Vascular modulation as a mechanism of estrogenic neuroprotection

The importance of vascular properties, such as vessel wall reactivity and contraction propensity, for the development of stroke is self-evident. Even though this category of factors may seem less important in animal models of cerebral ischemia where the vessel occlusion is artificial, it still influences the crucial aspects of collateral circulation and reperfusion. Consequently, it is likely that increased vasodilatation in the cerebral vascular bed is beneficial even in experimental cerebral ischemia by facilitating blood flow to compromised brain regions [188]. The reactivity and contraction propensity of a blood vessel is strongly influenced by locally produced vasodilators including prostacyclin and nitric oxide (NO), and vasoconstrictors such as endothelin-1, which in turn are regulated by other factors.

Estrogens have been shown to affect cerebral blood vessels in a number of stu-

dies; by relaxing cerebral arteries through inhibition of extracellular  $\text{Ca}^{2+}$  influx in vascular smooth muscle [189], moderating thrombotic mechanisms [190], influencing the biosynthesis of prostacyclin [191, 192], potentiating acetyl choline-induced endothelium-dependent relaxation [193], enhancing neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) levels [194-199] and thus increasing NO production [200-203], increasing cyclooxygenase-1 levels [195], and by less well characterized pathways which increase cerebral blood flow [32, 204-208]. It deserves mentioning that although eNOS could be neuroprotective through vasodilatation, it has also been shown to induce peroxynitrite formation under certain disease states [209], which in turn potentially could compromise cellular viability [210]. Most of these effects, such as influence on eNOS, cyclooxygenase-1 and prostacyclin synthase, leading to vasodilatation and improved collateral flow, seem to be exerted via the classical genomic pathway or via the PI3/Akt pathway [188].

In summary, substantial research efforts have been allocated to the elucidation of estrogens' mechanisms in stroke, however many unanswered questions remain, exemplified by the paradoxical effects on inflammation and oxidative stress.

## 1.4 Hormesis

The concept of hormesis reflects the pharmacological phenomenon that a substance can produce diametrically different effects depending on the dose, thus negating the notion that dose-response curves are generally unidirectional [211]. Although debated due to initially limited experimental evidence and lack of a clear definition, the concept has been successively established as a relevant model for explaining biological effects of certain substances [212].

### 1.4.1 Defining hormesis

The first record of the term "hormesis" in scientific publications is found in the 1943 article "Effects of extract of western red-cedar heartwood on certain wood-decaying fungi in culture" by Southam and Ehrlich. The authors investigated the effects of a wide concentration range of an anti-fungal agent, finding that despite high concentrations decreased the fungus growth, doses below the growth-inhibitory threshold actually stimulated it [213]. Thus, this original adoption of the term described the phenomenon that merely depending on the dose, one substance could have diametrically different effects in a biological system. However, although Southam and Ehrlich were the first to use the term "hormesis" in scientific publications, the phenomenon had been acknowledged much earlier. Actually, already the ancient Greeks' proverb "meden agan" (nothing in excess), the Latin analogue "in medio stat virtus" (virtue stands in the middle), as well as Paracelsus well-known quote "Alle Dinge sind Gift und nichts ist ohne Gift, allein die Dosis macht es, dass ein Ding kein Gift ist" (All things are poison and nothing is without poison; only the dose makes a thing not a poison) reflects aspects of hormesis. The scientist most often attributed to being the first to scientifically identify the hormetic phenomena, though without using the term "hormesis", was Schultz. In a series of studies as early as in the 1880's he demonstrated for

example that formic acid promoted fermentation in low doses while inhibiting it in higher doses [214].

Before and in parallel with the adoption of the term “hormesis” in the 1940’s, numerous terms for similar phenomena were suggested, including “biphasic”, “bidirectional”, “non-monotonic”, “J-shaped”, “U-shaped” and “inverted U-shaped dose-response curves”, “ $\beta$ -curve”, “Arndt-Schultz’ law” and “Huebbe’s law”. The rich flora of terms probably has contributed to confusion and difficulty in properly investigating the phenomenon. Therefore, the fundamental importance of clearly defining a term, such as “hormesis”, to precisely account for bidirectional dose-response relations of this sort cannot be overestimated.

A lively debate concerning the definition and significance of hormesis has taken place in the scientific community in recent years [211, 212, 215, 216]. One of the most influential scientists in the field is Calabrese, who has not only performed extensive literature analyses to assess the frequency and nature of the phenomenon [217, 218], but has also in a series of reviews revised the hormesis definition [212, 219-222]. An important contribution by Calabrese in developing a scientifically sound definition of hormesis was the realization that the low-dose effect of hormesis not necessarily is beneficial, since “beneficial” is an utterly complex and context-dependent denominator [212].

A related question is whether or not the mechanism(s) should be included in the definition of hormesis. In an attempt to more strictly define hormesis by attributing it to one common mechanism, it has been suggested that hormesis should be viewed as an adaptive action taken by the cell to minimize the damage from a toxic insult. This adaptation would in turn result from overcompensation due to the toxic damage, therefore with a mandatory time delay between insult and response [212, 219, 223]. However, it seems unnecessarily narrow to define one type of mechanisms for all types of hormetic dose-responses, as pointed out by Kendig et al. [211]. Further, such a definition is unintuitive, probably unrelated to many of the instances in which the term has been used, and the classification of a dose-response relation becomes exceedingly complicated if an adaptive nature of the response needs to be proven in every single case. Adaptation to toxic insults can definitely be one possible mechanism for certain hormetic responses, but the concept of hormesis should not be limited to this. Instead, Kendig et al. suggested that the definition of hormesis should solely be related to the bidirectional dose-response curve, and unrelated to its mechanism: “Hormesis is a dose-response relationship for a single endpoint that is characterized by reversal of response between low and high doses of chemicals, biological molecules, physical stressors, or any other initiators of a response” [211]. The advantage of this definition is that it is intuitive, readily enables identification of hormesis and is far less speculative than the mechanism-coupled definition. In line with this definition, Conolly and Lutz have illustratively demonstrated how different multi-mechanistic systems, including adaptations to damage, can render hormetic dose-response curves for certain endpoints. In this way it is highlighted that hormesis is most likely to occur in mechanistically com-

plex systems, where a multitude of mechanisms with different potency and efficiency taken together can create a bidirectional pattern [224].

It should be emphasized that not all non-monotonic dose-response curves are included in the concept of hormesis, but that effects in both directions compared to baseline need to be demonstrated (Figure 3)[225].

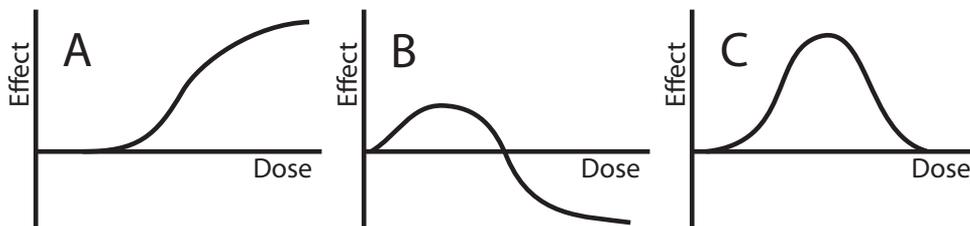


Figure 3. The classical linear/threshold dose-response relation is due to its monotonic behavior (A) clearly distinct from the non-monotonic hormetic pattern (B). However, not all non-monotonic dose-response relations are hormetic, exemplified by the unidirectional (producing effects on only one side of the baseline), non-monotonic relation presented to the right (C), which is not an example of hormesis.

The debate concerning the nature of hormesis has largely been conducted within the realm of toxicological sciences, essentially determining the nature of the suggestions as to how the term should be used. For example, the hormetic effect has most often been described as the sub-threshold stimulatory effect of a dose-toxicity curve, rather than for example the reversal of a drug's desired effect in doses above the therapeutic window [212]. It is worth emphasizing that the hormetic stimulatory window of toxic substances and the therapeutic window of pharmaceuticals are conceptually similar [211], and merely reflects different aspects of the same phenomenon. The dominating influence of toxicologists in the debate has probably also contributed to the widespread idea that the low-dose effect in hormesis is generally an adaptive response, an assumption that evidently makes most sense from a toxicological perspective.

Another matter of debate, which also needs to be addressed when using the term "hormesis", is its universality. The keenest proponents of hormesis have argued that hormesis is actually a more general phenomenon than the classical, well-established threshold theory, and should therefore be considered the default when assessing dose-response relations [212]. Although it seems plausible that hormetic phenomena are more common than hitherto demonstrated, and although advocating the search for hormesis by using wide ranges of concentrations is much deserving, it seems as yet unwarranted to claim that hormesis is universal since the phenomenon probably relies on different mechanisms in different instances and therefore is highly context-dependent. Moreover, the claim for its superiority to the threshold model and its generalizability has probably fuelled much of the recent skepticism towards the concept [211, 216].

Thus when in the remaining thesis referring to hormesis, we adhere to the defi-

nition suggested by Kendig [211], and view hormesis as a dose-dependent bidirectional effector-endpoint relation, which is unrelated to the mechanism and should not, although seemingly underappreciated, be considered universal.

#### 1.4.2 Examples of estrogenic hormesis

As mentioned earlier (section 1.2), estrogens exert their effects through multiple pathways, constituting a highly complex signaling system. In excess to the intricate signal pattern of nuclear and membrane receptors accounted for above (section 1.2), it has furthermore been speculated whether different subsets of membrane receptors, for example defined by their residence in membrane caveolae or lipid rafts, can result in non-monotonic dose-response relations [226]. These multifaceted signal systems in turn affect a wide range of biological mechanisms, further adding to the intricacy of estrogens' effects. Given this complexity, far from the single-receptor situation which is the conceptual basis of the linear dose-response model, it is not unexpected that estrogens theoretically could produce hormetic phenomena. As aforementioned (section 1.4.1), complex signal pathways is what mechanistically allow hormesis to occur [224] (Figure 4).

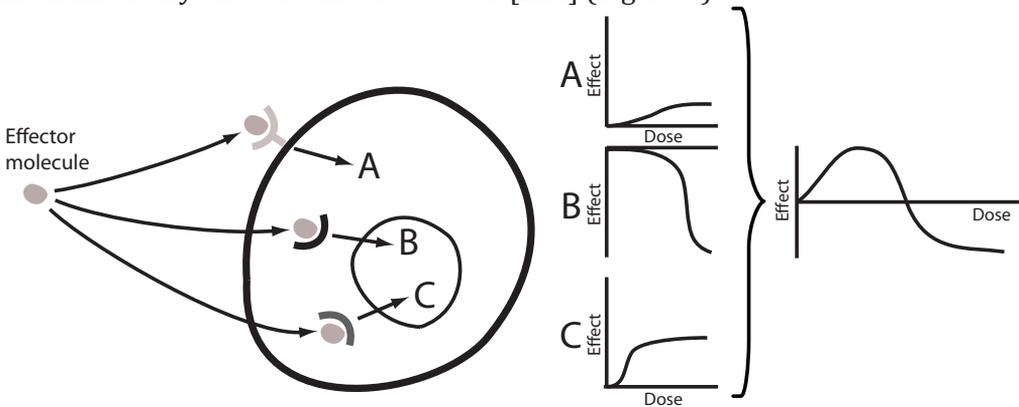


Figure 4. Given the fact that estrogens exert their effects through multiple pathways, differing in potency and effective concentration range, it is reasonable that when these are taken together, a more complex, for example hormetic, dose-response pattern can occur. (A), (B) and (C) correspond to different signal pathways in this hypothetical model, providing a theoretical, mechanistic framework for hormetic dose-response relations [225].

There are numerous examples of estrogenic hormesis affecting a wide variety of endpoints, including calcium content in bones [227], bone development [228], dopamine transporters and release [229], mammary gland differentiation [230, 231], capillary endothelial cellular adhesion [232], plasminogen activator regulation [233], DNA synthesis in endothelial cells [234], insulin sensitivity [235], genital development [236-238], growth of cultured tumor cells [239, 240] and multiple inflammatory processes [241-245]. A couple of these are presented below to further highlight the hormetic potential of estrogens.

##### 1.4.2.1 Estrogenic hormesis in inflammation

Many of the known examples of hormesis need pharmacological manipulation of the active substance to appear. However, when it comes to estrogens' effects on

inflammation, hormesis-like phenomena can actually be observed in vivo during pregnancy. Non-pregnant women are more T-helper cell type 1 (Th1)-tilted than men are, which has been assessed as an estrogenic effect, while the shift from Th1 to the antagonizing T-helper cell type 2 (Th2) appearing during pregnancy has also largely been attributed to changes in female sexual steroids [246]. Hence it seems that, even under physiological conditions, paradoxical suppression/potentialization of different parts of the immune system results from different concentrations of estrogens, which is compatible with the concept of hormesis. It is highly imaginable that pharmacological hormone manipulations even more potently can exert such phenomena. Numerous studies have been dedicated to experimentally investigate estrogens' effects on inflammation. The results reveal an almost inconceivable complexity, that however to a large part can be understood as consequences of the fairly logical overall effects of estrogens in pregnancy, aimed at avoiding abortion of the fetus [245]. Most experimental studies demonstrating hormetic phenomena of female sex hormones on inflammation suggest that low hormone concentrations are pro-inflammatory whereas high hormone concentrations are anti-inflammatory [242], such as the effects of estrogens on the pro-inflammatory cytokine IL-1 [247]. Similar results have been reported when it comes to the effects on TNF- $\alpha$  [241, 243, 244], natural killer cells and adhesion molecules, all seeming to be inhibited by high estrogen and/or progestagen levels while being stimulated by low levels [245]. Furthermore, inhibition of immune cell apoptosis has been demonstrated in lower levels than have the opposite [245]. These observations seem to be well in line with the understanding of the anti-inflammatory role of the high estrogen concentrations during pregnancy. However, the complexity increases when the effects of estrogens on a broader range of cytokines is taken into consideration, since not only concentration, but also the type of effector cell, the cytokine milieu and other factors seem to be crucial [245]. But even though estrogens' actions on inflammation in different organs and cells are exceedingly complex and it is difficult to draw any firm conclusions, it is clear that estrogens are highly capable of exerting hormetic effects in inflammation, and that this needs to be taken into account when studying relevant phenomena. Hormetic effects on inflammation are particularly interesting since they are relevant for stroke and not only for more obviously inflammatory disorders such as rheumatoid arthritis.

#### 1.4.2.2 Estrogenic hormesis in cardiovascular disease

Hormetic effects of estrogens on inflammation also have far-reaching implications for a broader range of cardiovascular diseases, since inflammation for example is a central process in the pathogenesis of atherosclerotic plaques [248] and in the development of myocardial infarction [249]. However, there are more specific examples of hormesis that can be relevant to cardiovascular disease, such as the effects on the anticoagulant protein plasminogen activator. In a cell culture experiment using bovine aortic endothelial cells the effects of 17 $\beta$ -estradiol on plasminogen activator was investigated. It was found that even though 17 $\beta$ -estradiol concentrations corresponding to low physiological

in vivo levels activated the protein, higher concentrations inhibited it, and thus it was concluded that in this respect, estrogens in pharmacological doses can be thrombogenic [233]. Further, estrogens' effects on the DNA production in endothelial cells have been reported to obey hormetic principles. In a human umbilical smooth muscle cell line it was found that  $17\beta$ -estradiol in physiological concentrations stimulated [3H]thymidine incorporation into DNA whereas pharmacological concentrations were inhibitory. These findings may have bearing on cardiovascular diseases because of the role of smooth muscle cells in atherosclerosis pathophysiology [234]. Moreover, insulin resistance is a prominent feature of the metabolic syndrome and thus intimately related to cardiovascular diseases. In a randomized controlled trial of the effects on conjugated equine estrogens on insulin sensitivity in postmenopausal women it was shown that the standard dose of 0.625 mg/day increased while 1.25 mg/day decreased insulin sensitivity [235]. This is a clear demonstration that the hormetic effects of female sex hormones can indeed prevail in clinical situations.

## 2. Hypothesis

**T**he main hypothesis is that the dichotomous effects of estrogens on ischemic stroke can be explained by methodological differences, especially in the choice of hormone administration regimens, resulting in different serum concentrations of 17 $\beta$ -estradiol.

## 3. Aims

- To characterize the three most well-used  $17\beta$ -estradiol administration methods to rats and to design a useful and attractive peroral alternative.
- To investigate which methodological parameters were responsible for the discrepancies in results in previous studies investigating the impact of estrogens on focal cerebral ischemia in rats, with a focus on  $17\beta$ -estradiol administration methods and dose.
- To test how altering  $17\beta$ -estradiol administration methods and doses affects the impact of estrogens on ischemic stroke in rat models.

## 4. Materials and Methods

### 4.1 Overview of study designs

The aim of Paper 1 was to investigate the serum concentrations produced by the three most commonly used  $17\beta$ -estradiol administration methods, namely subcutaneous silastic capsules, slow-release pellets and daily injections. Further, the effect of a two-week washout period was assessed, and two pellet concentrations were tested. After ovariectomy and washout, the animals were administered the  $17\beta$ -estradiol. Blood samples were obtained from the  $17\beta$ -estradiol treated groups on days 2, 7, 14, 21, 28, 35 and 42. An ovariectomized control group and three native control groups, studied in different estrous cycle phases, were included as well. The animals in the control groups were decapitated and blood samples collected from the trunks. All samples were analyzed by means of  $17\beta$ -estradiol radioimmunoassays (RIAs) (Figure 5).

In Paper 2, we used a meta-analytical approach to investigate the impact of different methodological parameters on the effect of estrogens on stroke. After 14 parameters had been identified, information from all available articles that had tested the effect of estrogens on stroke in rats was extracted and analyzed (Figure 5).

The objective of Paper 3 was to replicate an earlier stroke study from our lab in all aspects except the administration regimen; silastic capsules were to be used instead of slow-release pellets. The hypothesis was that this would make the  $17\beta$ -estradiol protective instead of detrimental. We also aimed to investigate the effect of pretreatment only and of only administering at the time of MCAo. Thus four groups were included, receiving placebo during the entire experiment,  $17\beta$ -estradiol during the entire experiment,  $17\beta$ -estradiol only prior to MCAo and  $17\beta$ -estradiol only from start of MCAo. Rats were ovariectomized and administered placebo or  $17\beta$ -estradiol. Fourteen days later, all rats were inflicted focal cerebral ischemia via MCAo, and placebo/hormone treatment was adjusted according to the group protocols. Another three days later, rats were decapitated and brains analyzed for infarct size (Figure 5).

Paper 4 was in many aspects similar to Paper 1, except in regard of the time perspective, since Paper 4 focused on the first two days after administration. The same administration methods and a novel peroral method were tested, but each administration group was divided into two subgroups to allow the frequent blood sampling procedures. Following ovariectomy and (except in one of the silastic capsule groups) two weeks of wash-out,  $17\beta$ -estradiol was administered and blood samples drawn at time-points 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h and 7 days from the two subgroups alternatively. The control groups were also similar to Paper 1, with an ovariectomized and two native control groups. Further, the novel peroral method was tested in a long-term perspective, according to the experimental protocol in Paper 1 (Figure 5).

For Paper 5, 60 animals were divided into three groups of 20; one vehicle group, one low-dose  $17\beta$ -estradiol group and one high-dose  $17\beta$ -estradiol group. Fourteen days after ovariectomy and hormone administration, animals were inflicted focal cerebral ischemia, and decapitated three days later for infarct size analysis. Blood samples were drawn days 2, 7, 14 and 17, and functional tests were performed days 14, 15 and 17. The animals, their food and their water were weighed on days 2, 4, 7, 10, 14 and 17. A sham group underwent the same treatment except that the ovariectomy and MCAo were sham-procedures (Figure 5).

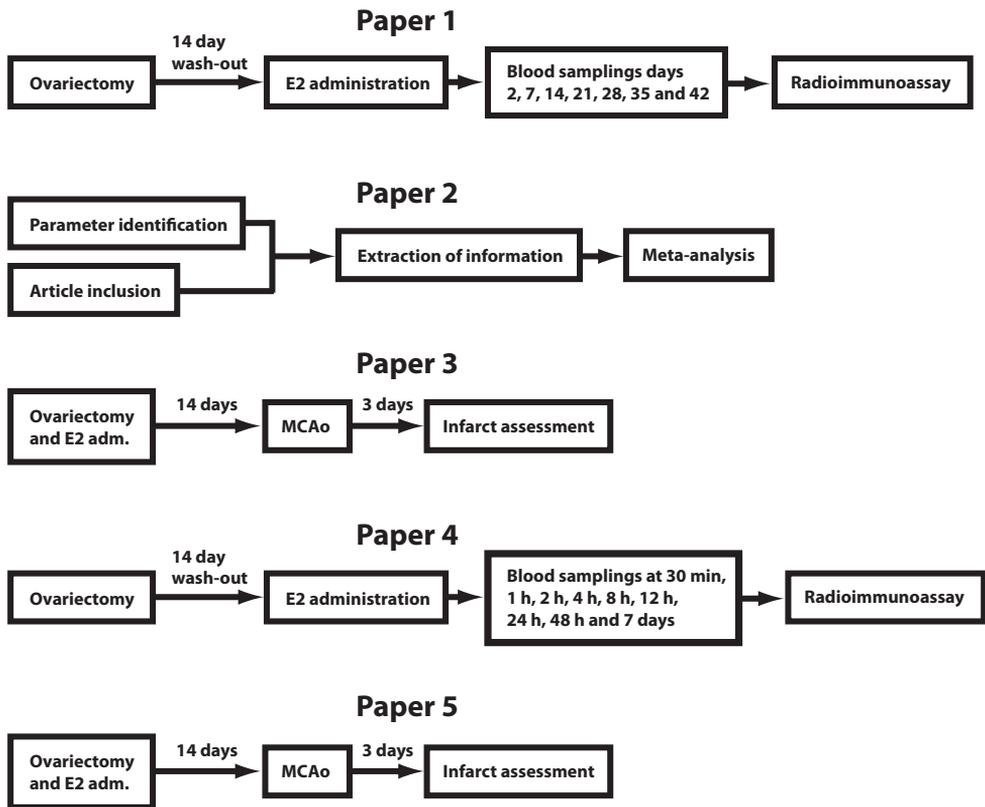


Figure 5. Simplified overview of study designs behind Papers 1 to 5.  $17\beta$ -estradiol is abbreviated "E2" in the figure.

## 4.2 Animals

Surgery to induce ischemic stroke is relatively invasive, and infections are not uncommon, which is one reason why rats are more popular than the less robust mice. Also, the microsurgical techniques become even more demanding in smaller animals, and rats are generally considered more intelligent than mice and thus easier to perform functional tests on. The Sprague-Dawley rat strain is very commonly used, and is the dominant animal model in the estrogen-stroke field. It is an albino strain, originating from the Sprague Dawley Company, Madison, Wisconsin, USA, in 1925 (Harlan Laboratories' homepage, [http://www.harlan.com/products\\_and\\_services/research\\_models\\_and\\_services/research\\_models/](http://www.harlan.com/products_and_services/research_models_and_services/research_models/)

sprague\_dawley\_outbred\_rat.hl, accessed 2012-02-14). Since it is outbred, the strain is more genetically heterogeneous than for example Spontaneously Hypertensive Rats, however the difference in pricing between inbred and outbred strains is, in our opinion, too large to justify the use of inbred animals.

The reason that we in all our studies used female animals is that we investigated the effects of estrogens, which could have implications for hormone therapy in menopause. Evidently, female rats are more similar to women than are male rats, making a female animal model more relevant.

In total, 413 female Sprague-Dawley rats were used in the experiments behind the current thesis: 120 in Paper 1, 48 in Paper 3, 175 in Paper 4 and 70 in Paper 5. All procedures were conducted in accordance with the National Committee for Animal Research in Sweden and Principles of Laboratory Animal Care (NIH publication no. 86-23, revised 1985). The studies were approved by the Local Ethics Committee for Animal Care and Use at Linköping University.

### **4.3 Ovariectomy and 17 $\beta$ -estradiol administration**

When studying the effects of estrogens in female rats, the hormones' natural cyclicity can be a confounding factor. In attempt to control the sex hormone levels it is common practice to remove the main endogenous source via ovariectomy, to then substitute with 17 $\beta$ -estradiol. Concerning the last step – the administration of 17 $\beta$ -estradiol – there is a profound lack of consensus regarding what procedure is optimal, and numerous methods have been adopted in different studies. Several attributes are demanded from a good hormone administration regimen; the method should be cheap, possible to standardize, easily used and should also minimize the handling of the animals. Another central aspect is the serum hormone concentrations produced by the regimen. Stable, physiological (or sometimes pharmacological) concentrations are generally wanted when testing the effect of the hormone on biological systems. Since 17 $\beta$ -estradiol has a short half-life in the body [13] the production of a stable level of hormone relies on slow release from the injection/ implantation site (alternatively the complicated and demanding process of continuous infusion of the hormone).

In the studies in the current thesis, all rats except native controls were ovariectomized. Three of the most well-used methods for administering 17 $\beta$ -estradiol to ovariectomized female rats – commercial slow-release pellets, subcutaneous injections and home-made silastic capsules – were tested in Papers 1 and 4, and silastic capsules were used in Papers 3 and 5. Further, a novel peroral method in which the 17 $\beta$ -estradiol was administered via the hazel-nut spread Nutella® (Ferrero Scandinavia AB, Malmö, Sweden) was evaluated in Paper 4. The ovariectomy procedure and administration methods are described in detail below.

#### **4.3.1 Ovariectomy**

Ovariectomies were performed via the dorsal route. The anesthetized animal was put in prone position, and a 3 cm dorsal midline incision was made cephally

starting from the level of the iliac crest. The skin was bluntly dissected from the underlying fascia, after which the fascia was incised, creating two 1 cm long apertures on each side of the spine, 1 cm from the midline. Subsequently, the abdominal cavity was reached via careful blunt dissection under the fascia, revealing the adipose tissue in which the ovary was embedded. The ovary was gently pulled out, ligated and cut, and the stump put back into the abdominal cavity. The same procedure was performed through the contralateral fascia aperture. Then the fascia incisions and skin wound were closed by 4-0 vicryl sutures.

### 4.3.2 *Slow-release pellets*

The slow-release pellets tested in Papers 1 and 4 were manufactured by the company Innovative Research of America (IRA, Sarasota, FL 34236, USA), and consisted of 17 $\beta$ -estradiol, cholesterol, cellulose, lactose, phosphates and stearates (<http://www.innovrsrch.com/faq.asp#R1>, accessed 2008-08-18). This kind of pellet has been well-used in animal experiments [250-253] and has the obvious advantages that it is standardized (produced by only one company), easy to use and only requires one-time handling of the animal. The manufacturer claims that they yield a constant release of hormone once steady-state is achieved, which is stated to occur within 48 h. The pellets are administered by implanting them in the subcutis. For this, the anesthetized animal is put in prone position and a 1 cm incision is made in the loose skin of the rat's neck. After a pocket has bluntly been dissected caudolaterally, the pellet is gently installed and the incision is closed.

Two types of IRA pellets were selected for the testing in Papers 1 and 4, containing 0.25 mg and 0.1 mg 17 $\beta$ -estradiol, designed to release 4.2 and 1.1  $\mu$ g 17 $\beta$ -estradiol/day respectively (60-day release, SE-121, 0.25 mg/pellet and 90-day release, NE-121, 0.1 mg/pellet, IRA). These were chosen on the basis that they were supposed to release amounts of hormone in the same range as we had chosen for our injection regimen (10 $\mu$ g/kg body weight/day) in the same studies. Also, in comparison to earlier studies from our laboratory we chose lower doses of 17 $\beta$ -estradiol in hope of obtaining more physiological serum concentrations [1]. In the pilot study to Paper 5, the same potent pellets (90-day release, NE-121, 1.5 mg/pellet, IRA) that had earlier been used by our group [1] were tested in an effort to design a home-made silastic capsule that rendered equally high serum hormone concentrations (Figure 6A; Figure 7B and C).

### 4.3.3 *Subcutaneous injections*

The method of subcutaneous 17 $\beta$ -estradiol injections have been widely used in rat experiments [82, 254, 255] and have its greatest advantage in being readily accessible in almost any laboratory, easy to use and also easily described and standardized. A major drawback, except for the produced serum profile as described below, is that it requires daily animal handling, which can be quite stressful for the rats.

The regimen tested in Papers 1 and 4 was one single 17 $\beta$ -estradiol injection of 10 $\mu$ g/kg body weight/day (CAS No. [50-28-2], Sigma-Aldrich Sweden AB, Stock-

holm, Sweden) dissolved in 30 mL of sesame oil (CAS No. [8008-74-0], Sigma-Aldrich Sweden AB, Stockholm, Sweden). This is a frequently used injection dosage to achieve physiological levels of  $17\beta$ -estradiol [82, 254]. A Hamilton® syringe (1700 series, 100 mL, Sigma-Aldrich Sweden AB, Stockholm, Sweden) was used for injections. Before administration, the solution was stirred with a magnetic stirrer for at least 20 h. Injections were done in the rat neck subcutis, between 10 and 12 a.m. every day (Figure 6B; Figure 7D and E).

#### 4.3.4 Silastic capsules

Silastic capsules have been used for administration of hormone to experimental animals in numerous studies [256-260]. The silastic membrane of the capsule functions as a semi-permeable barrier, prolonging the release of hormone from the implantation site. The advantages are that the method is cheap, adaptable and only require one-time handling of the animal. Since the capsules are “home-made”, many factors can be varied (length and width of silastic capsule, sealing, hormone concentrations, incubation time, incubation solution etc.). Hence, the alternatives in how to produce the capsules are infinite, and thus it is pivotal to always exactly describe the capsule design.

The capsules used in Papers 1, 3, 4 and 5 were assembled as described by Wise et al. and have earlier been used in studies showing  $17\beta$ -estradiol neuroprotection [261]. Thirty millimeter segments of silastic laboratory tubing (Inner/outer diameter: 1.575/3.175 mm, Dow Corning, VWR International, Buffalo Grove, IL, USA) were filled with a solution of  $17\beta$ -estradiol in sesame oil, or sesame oil only in the placebo groups. In all estrogen capsules in the included articles the concentration of  $17\beta$ -estradiol was 180  $\mu\text{g}/\text{mL}$ , except in the high dose group in Paper 5, containing 50 000  $\mu\text{g}/\text{mL}$ . Five millimeter pieces of wooden applicator sticks (birch, length 15 cm, diameter 2 mm, SelefaTrade AB, Spånga, Sweden) were cut using a fine tooth saw and used to seal the silastic tubing, resulting in an oil- $17\beta$ -estradiol-filled column 20 mm in length. The capsules were stored overnight in a vial containing sesame oil with the same concentration of  $17\beta$ -estradiol as inside the capsules. Before implantation, the capsules were carefully wiped. A 0.5-1 cm incision was made in the loose skin of the rat's neck, and a pocket was bluntly dissected caudally, in which the silastic capsule was gently installed using forceps. The incision was subsequently closed by a suture (Figure 6C; Figure 7F and G).

#### 4.3.5 Peroral method

Providing peroral  $17\beta$ -estradiol to the animals has the advantage of imitating hormone consumption in women. Because of differences in absorption rates and first pass metabolism, mimicking the exact administration routes and doses may be highly relevant when constructing animal models that are supposed to be similar to the clinical situation. To that effect we designed a peroral administration regimen, in which the  $17\beta$ -estradiol was mixed in Nutella®, to be tested in Paper 4. Nutella® has previously been used for administration of buprenorphine to mice [262], but to our knowledge never for hormone administration.

The rats were trained to eat Nutella® for five days before the experiment, first in groups of five once a day for three days. This was performed by placing 2 g of hazelnut cream on a 5x5 cm ceramic tile in the cage. Once a day the following two days the rats were placed in separate cages before receiving 0.5 g of hazelnut cream each. The day before commencing the hormone administration, 17 $\beta$ -estradiol dissolved in sesame oil was mixed with the chocolate flavored hazelnut cream. Prior to each administration, the rats were placed in separate cages to ensure that each rat got its individual dose of 28  $\mu$ g 17 $\beta$ -estradiol dissolved in 5  $\mu$ L of sesame oil and 1 g of hazelnut cream/kg body weight/day, corresponding to a 2 mg 17 $\beta$ -estradiol dose to postmenopausal women. The estrogen mixture was placed on the 5x5 cm ceramic tiles and administered to the rats between 8.00-10.00 a.m. each day (Figure 6D; Figure 7H and I).

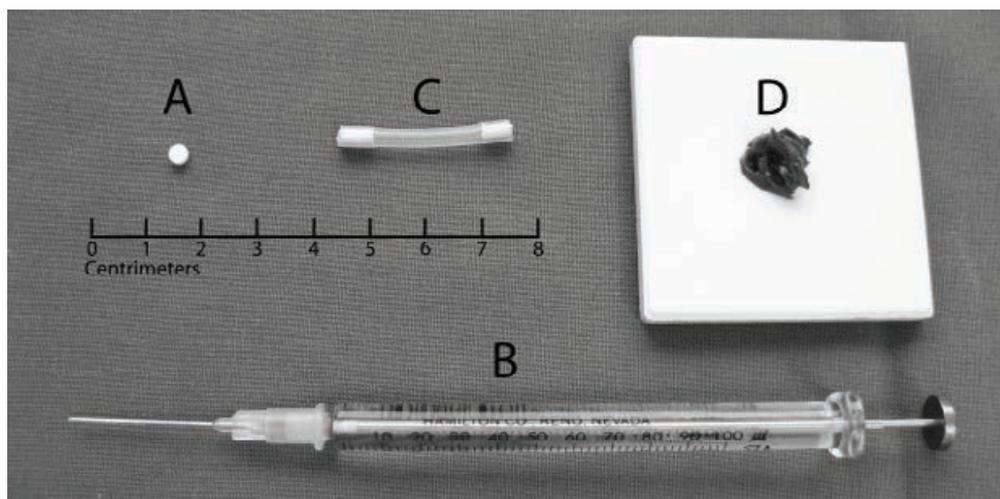
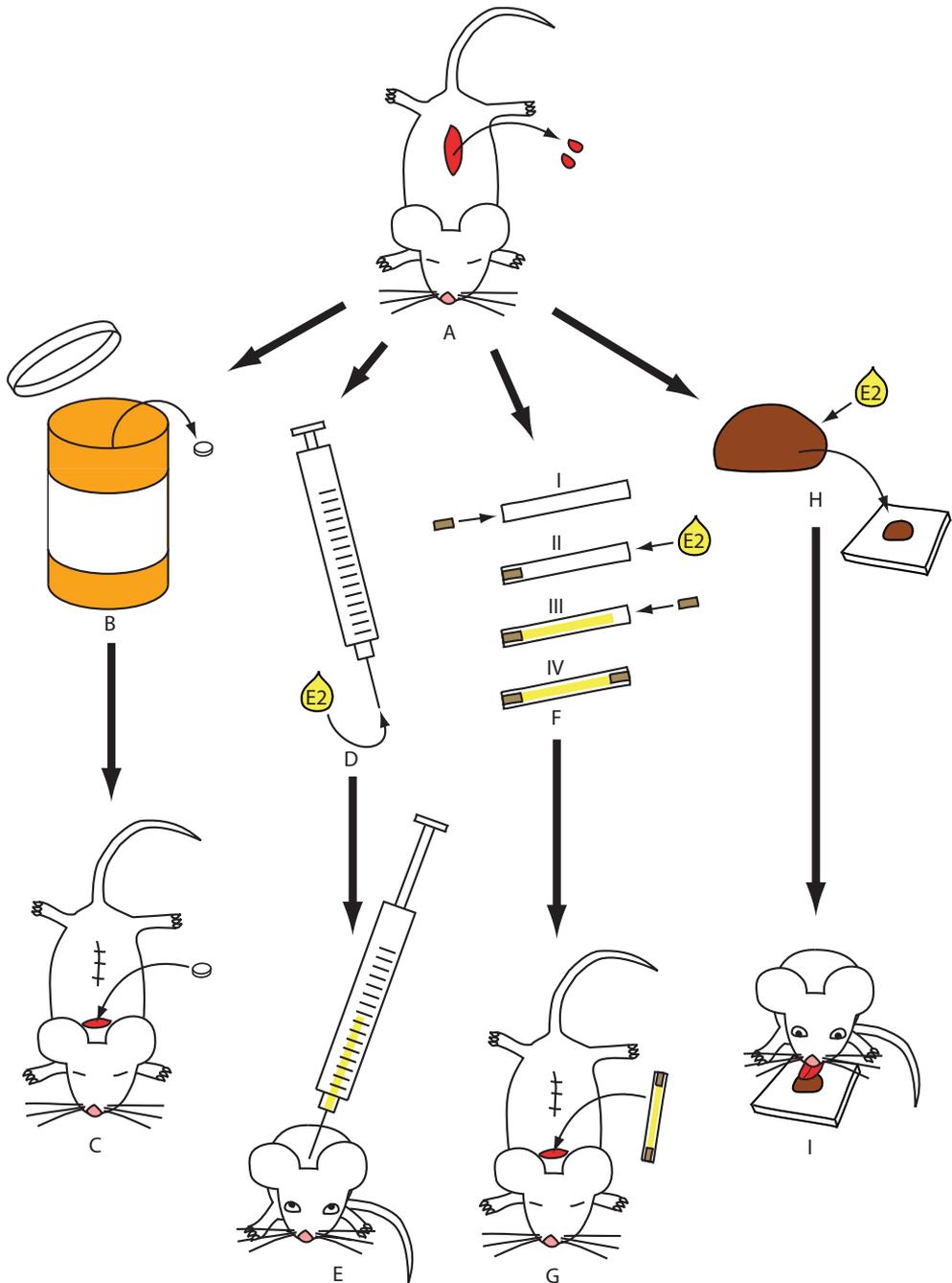


Figure 6. Photo of 17 $\beta$ -estradiol administration methods to rats: (A) slow-release pellet from IRA, (B) 100  $\mu$ L Hamilton® syringe for subcutaneous injection, (C) silastic implant and (D) the hazelnut spread Nutella® served on a ceramic tile.

Figure 7 (to the right). The first step of the estrogen supplementation procedure is to remove the ovaries, which may be done two weeks prior to hormone administration. If pellets (B) are used, they are implanted subcutaneously in the loose neck skin of the anesthetized animal (C). Daily subcutaneous injections (D) are another alternative, and can be done in the neck skin of an awake animal (E). Silastic capsules (F) are prepared by the following procedure: (FI) one end of the silastic tube is plugged with a piece of wooden applicator stick, (FII) 17 $\beta$ -estradiol in sesame oil is injected into the tube and (FIII) the other end is plugged, resulting in a sealed hormone container (FIV) to be installed subcutaneously in the anesthetized animal, in the same manner as the pellets (G). The peroral Nutella® method is prepared by mixing 17 $\beta$ -estradiol in sesame oil with the hazelnut spread (H). The mixture is subsequently served to the animal on ceramic tiles, repeated daily (I).



#### 4.4 Blood sampling

Blood samples were obtained for hormone assays in Papers 1, 3, 4 and 5 through venipuncture of the hindleg or via decapitation. To perform the venipuncture, the anesthetized animal was put in prone position, the leg was shaved and a rubber band was tied around the extremity to visualize the saphenous vein. Then

the vein was punctured using a 23 gauge needle (Sterican<sup>®</sup>, Braun Medical AG, Emmenbrücke, Switzerland), and the blood collected into a 1 mL syringe (BD Plastipak<sup>™</sup>, Becton Dickinson SA, Madrid, Spain). It was subsequently put in a serum clot activator tube (Vacuette<sup>®</sup> Serum Tubes; Hettich Labinstrument AB, Sollentuna, Sweden), centrifuged and stored in -20°C until analysis. For the decapitation samples, the blood running from the trunk was collected into a 10 mL laboratory plastic tube, centrifuged and stored in -20°C pending analysis.

### 4.5 Middle cerebral artery occlusion

The goal of the MCAo procedure is to cause focal cerebral ischemia by obstructing the blood flow to a part of the brain. There are many ways in which this can be achieved, and the two most commonly used ones are the intraluminal filament method [263] and the direct occlusion method [264]. During the work with this thesis, we switched from the direct method, used in Paper 3, to the filament method, used in Paper 5. There were multiple reasons for this method change, one being that the large intragroup variation was a constant concern with the direct method. Other drawbacks with the direct method are that on the level of exertion it is an intricate practice, and on the level of possible side-effects it requires a craniotomy, which could impact the subsequent brain pathology. Further, demonstrating consistent findings in different models increases the likelihood that a phenomenon is biologically relevant.

In Paper 3, all animals underwent MCAo according to the following procedure. The rats were anesthetized and placed with their left side up on a thermostatic heating pad (Harvard Homeothermic Blanket System, Edenbridge, UK) to maintain the core/rectum temperature at 37.0±0.5°C. The left femoral artery was cannulated using a soft catheter Micro-renathane<sup>®</sup> tubing (MRE-025 Bintree Scientific Inc., MA, USA) primed with saline containing heparin (100 IU/mL, Løvens, Ballerup, Denmark) for registration of blood pressure and pulse (Acq-Knowledge software, BioPac system, Goleta, CA, USA; and Blood Pressure Transducer 56360, Stoelting, IL, USA). The left MCA was temporarily occluded during 60 min using a microclip. Using an operating microscope (Zeiss Opmi 6-H, West Germany), the left MCA was exposed transcranially [264] removing the parotid gland and part of the zygomatic bone but keeping the temporalis muscle and the facial and mandibular nerves. The MCA was occluded with a microclip between the rhino cortical branch and the lenticulostriate artery [265]. After one hour, the clip was removed, the wound closed and the animal allowed to wake up (Figure 8).

The animals in Paper 5 were, as stated above, inflicted focal cerebral ischemia by the intraluminal filament method, based on the method described by Longa [263]. The anesthetized animal was put in supine position, and after shaving, a 2 cm cervical midline incision was made. The common (CCA), internal (ICA) and external (ECA) carotid arteries were freed from surrounding tissue. CCA and ECA were ligated with a suture (6-0 silk suture, Johnson & Johnson, New

Brunswick, NJ, USA), while ICA was temporarily clipped with a vascular microclip (8 mm artery clip, Rebstock Instruments GmbH, Dürbheim, Germany), after which a small incision was made in the CCA. Then a 30 mm silicone coated 4-0 nylon suture (403756, Docol, Redlands, CA, USA) was inserted and advanced up ICA approximately 18-20 mm until a light resistance was felt, indicating correct placement. The filament was secured by a knot and the animal allowed waking up. After 60 minutes of occlusion, the rat was reanesthetized, the filament was withdrawn and ICA was permanently ligated. The rats were let to recover in heated ( $25\pm 2^{\circ}\text{C}$ ) cages for one hour, and during the first 24 h postoperatively, water-soaked food pellets were placed in a petri dish on the cage floor to promote eating (Figure 8).

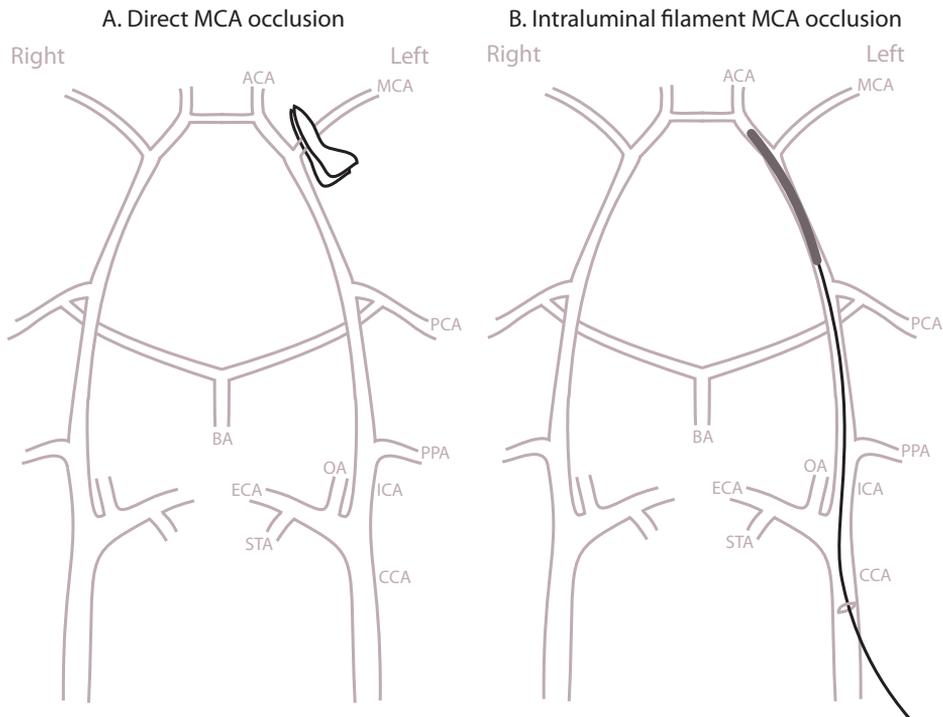


Figure 8. The method of direct MCAo (A), used in Paper 3, is based on the straightforward principle of putting a clip on the MCA. This is achieved by careful dissection through the skull and further inwards under the brain to visualize the artery. The intraluminal filament method (B), used in Paper 5, does not require a craniotomy. Instead, the MCA is occluded by introducing a filament (in the figure with a silicone coated tip) into the CCA, which only requires a midline throat incision. Abbreviations for not previously mentioned vessels: anterior cerebral artery (ACA), posterior cerebral artery (PCA), pterygopalatine artery (PPA), basilar artery (BA), occipital artery (OA) and superior thyroid artery (STA).

## 4.6 Outcome measures

### 4.6.1 Radioimmunoassays

A methodological fundament for comparing methods of hormone administration is the assessment of serum hormone levels. The RIA technique combines the specificity and accuracy of biological antibodies with the possibility of measuring very low numbers of radioactive molecules in a highly sensitive method that can detect concentrations of for example  $17\beta$ -estradiol in the picomolar range. The method has for a number of years been readily accessible in the form of commercial “direct” RIA kits. Since these kits are designed to be used without a preceding extraction step and everything needed is included in the package, they are very convenient and thus widely employed, not least in rat experiments [252, 266, 267].

#### 4.6.1.1 $17\beta$ -estradiol analysis

The commercially available DPC  $^{125}\text{I}$  RIA kit ( $17\beta$ -estradiol double antibody, KE2D, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) was used in Papers 1, 3, 4 and 5. This method has, according to the manufacturer, a lowest detection limit of 1.4 pg/mL and intra- and inter-assay coefficients of variation of 4-13 % and 3.5-5.5 %, respectively, depending on the concentration range. In all studies, several kits were needed due to the large number of serum samples to be analyzed. Different kits were of the same LOT-number within each study, and before the analysis, the reagents were pooled to avoid inter-assay variation. Standard curves were analyzed at least every 100 samples, and all samples were read against their closest standard curve. The serum volumes used in each tube, both for standard curve and samples, were 100  $\mu\text{L}$  in Papers 1 and 3, and 50  $\mu\text{L}$  in Papers 4 and 5. The performance of the most deviating of these protocols, the 50 $\mu\text{L}$  variant, was compared to the original protocol (with 200  $\mu\text{L}/\text{tube}$ ), showing a linear correlation of  $r=0.995$  and a Deming's orthogonal regression formula of  $y=0.931x+1.32$ . Standards and samples were analyzed in duplicate and measured for 300 seconds in a gamma counter (Gamma Master 1277; Wallac-Pharmacia, Turku, Finland).

#### 4.6.1.2 Estrone sulfate analysis

Estrone sulfate is a hepatic metabolite of  $17\beta$ -estradiol, that because of first pass metabolism theoretically could be higher in peroral than subcutaneous administration regimens. To test this hypothesis, estrone sulfate was analyzed in Paper 4 using a commercially available  $^{125}\text{I}$  RIA kit (Estrone sulfate double antibody, DSL-5400, Beckman Coulter, Brea, CA, USA) and measured for 400 seconds in the gamma counter mentioned above (section 4.6.1.1). According to the manufacturer, this method has a lowest detectable concentration of 10 pg/mL, and intra- and inter-assay coefficients of variation of <9.2 % and <8.8 %, respectively. All kits used were of the same LOT number and reagents from the different kits used were pooled before analysis. Standards and samples were analyzed in duplicate and 100  $\mu\text{L}$  of serum was used in each tube.

#### 4.6.2 Infarct size analysis

In Papers 3 and 5, staining with 2,3,5-triphenyltetrazolium hydrochloride (TTC; Cas No. [298-96-4], Fluka Analytical, Sigma-Aldrich Sweden AB, Stockholm, Sweden) was performed for infarct size analysis. TTC stains viable mitochondria red, leaving infarcted tissue white. It is a convenient method since it can be used on fresh and relatively thick (2 mm) tissue slices, and requires no microscopic evaluation.

The rats were anesthetized by pure carbon dioxide and sacrificed by means of a rat guillotine. The brain was carefully dissected out and cooled in +4°C saline. Two millimeter thick coronary slices of the brain were cut with razor blades directed by a rat brain matrix (RBM-4000, ASI Instrument Inc., USA). The slices were soaked for 10 min (20 min in Paper 5) in a solution of 2 % TTC in 0.1 mol/L phosphate-buffered solution (pH 7.4) in a small Petri dish, maintained at 37°C in a heater. Gentle stirring of the slices ensured even exposure of the surfaces to staining. Excess TTC was then drained, and the slices were scanned (ScanJet 2c, Hewlett-Packard, Palo Alto, CA, USA). The size of brain lesion was measured using SigmaScan Pro version 5 (SPSS, IBM, Armonk, NY, USA) using an automatic threshold of 40 % in the green spectrum in a similar manner as described by Bederson et al. and Goldlust et al. [268, 269]. In Paper 3, infarct sizes were presented as slice areas and were not corrected for edema. In Paper 5, the lesion volumes were calculated, and edema correction according to the following formula was performed:

$$[\text{Infarct size}] * [\text{Total contralateral hemisphere}] / [\text{Total ipsilateral hemisphere}]$$

#### 4.6.3 Uterine weight

The uterus is affected by the circulating estrogen concentrations, and has therefore in many instances been used as a surrogate measure for 17 $\beta$ -estradiol serum concentrations [270, 271]. To evaluate the adequacy of such an approach, uterine weights were assessed in parallel with the 17 $\beta$ -estradiol serum concentrations in Paper 4.

Immediately after sacrifice an abdominal midline incision was made and the uteri removed. Since the uterine horns were truncated to a varying degree during the preceding ovariectomy, the entire uterus weight depended on the length of the cut horns. Therefore, to allow a standardized measure of uterine weight, only one centimeter of each uterine horn was cut out and weighed.

#### 4.6.4 Subcutis histology

Implanting material or injecting substances subcutaneously bears the risk of causing local reactions, which could be a problem if the intended outcome measure is affected. To address this concern, the subcutis from administration sites in the animals in Paper 4 was sampled. Immediately after the sacrifice, samples of subcutaneous tissue were cut out from the administration site in the neck of three rats each in the relevant groups. The tissue samples were prepared and stained with eosin-hematoxylin. They were then assessed by a pathologist that was blinded in regard to the samples' group affiliations.

### 4.6.5 Food and water weight

Since estrogens affect metabolism and food consumption, and given the fact that starvation or over-feeding theoretically could affect infarct sizes, it was decided to assess food and water consumption in Paper 5. On days 0, 2, 4, 7, 10, 14 and 17, the rats, their food and their water were weighed, and daily consumption for the rats in each cage was calculated. A difficulty with this procedure was that since specially designed food measurement cages were not used, any food and water that was spilled had to be neglected.

### 4.6.6 Functional tests

There are numerous methods for extending the relevance of treatments for focal cerebral ischemia beyond decreased infarct sizes. The functional tests chosen for Paper 5 – the tail swing test and the cylinder test – have the advantages of requiring almost no equipment, being easy to learn and also require no training of the animals.

The rats underwent the tail swing test and cylinder test prior to surgery, and 1 and 3 days postoperatively. For the tail swing test, the animal was lifted in the tail, and the direction of the first 20 attempts to reach the experimenters hand (swings) were recorded, so that a right-left index could be calculated [272]. For the cylinder test, the rat was put in a glass beaker (diameter: 18 cm, height: 26 cm), and the 10 first times the rat reared, the use of the two paws to touch the glass was recorded, also resulting in a right-left index [273].

## 4.7 Anesthetics, analgesics and perioperative surveillance

During surgical procedures, it is of utmost importance to ensure that the animal suffers as little as possible. The reason for this is mainly ethical, but caring for the animal's comfort also has scientific implications. An animal in pain will for example have a high sympathetic drive and eat less, which could influence the result and be a source of error.

For the ovariectomies and blood samples in Papers 1, 3, 4 and 5, and the MCAo procedures in Papers 3 and 5, anesthesia was induced by 4.2-4.5 % isoflurane (Forene® inhal-v 250 ml, Abbott Laboratories. Abbott Park, IL, USA) in a mixture (30 %/70 %) of oxygen/nitrous oxide in an induction chamber. For maintenance, 1.4-1.5 % of isoflurane in (30 %/70 %) of oxygen/nitrous oxide was provided through a face mask, except during MCAo in Paper 3, where the animals were intubated and maintained on 1.2-1.4 % isoflurane (Zoovent®, CWC600AP, ULV Ltd., UK). For the intubated animals, the tidal volume and ventilation frequency were carefully regulated using on site monitoring of blood gases and acid/base status (AVL, OPTI 1 Medical Nordic AB, Stockholm, Sweden).

During all surgical procedures, eye gel (Lubrithal® 15 g Leo laboratories Ltd., Dublin, Ireland) was utilized for protection of the rats' eyes. During the ovariectomies, the animals were also injected with 5 mg Rimadyl®/kg body weight (50

mg/mL Pfizer, Dundee, Scotland) dissolved in 1 mL of saline subcutaneously in the neck, while lidocain gel (Xylocain 2 %, AstraZeneca AB, Södertälje, Sweden) was applied in the wound during filament MCAo in Paper 5. During all surgical procedures, body temperature was maintained by a heating pad connected to a rectal thermometer.

## 4.8 Data acquisition in Paper 2

The goal of the meta-analysis in Paper 2 was to investigate the effect of different methodological parameters on the impact of estrogens on focal cerebral ischemia. To do this, we attempted to find all original experimental studies published until July 2008 that used a relevant experimental setup. Medline, Embase and Scopus were searched using the keywords “rat”, “estrogens” or “estradiol”, “cerebral” and “ischemia”. The reference lists of thirty review articles were searched for additional original studies [168, 188, 274-301], and finally the reference lists of all original studies were searched. The earliest study included for analysis was from November 1997 [31].

For a study, or a particular set of experimental groups within a study, to be included in the current analysis, the following was demanded:

- The experiments must be conducted in rats.
- Estrogen administration must be the variable differing between the experimental groups, with no additional drugs administered.
- The cerebral injury must be mainly ischemic, and physically, not for example pharmacologically, induced.
- The outcome measure must be directly related to the damage, for example size of the cerebral ischemic lesion, neuronal cell loss in hippocampus or neurological deficits and not for example blood-brain barrier disruption or endothelial dysfunction. Also, the result must be quantitatively accounted for in the text, a table or a graph.
- Each estrogen treatment group must have a corresponding placebo group.

The study was restricted to one species – rats – since 1) they had been most extensively used for experimental stroke studies, 2) there were unexplained differences between research groups in the results and conclusions of these studies and 3) since we had – both experimentally and by studying the results of others – reasons to believe we had found an explanation for the dichotomy of results in rat studies.

Data from all included articles were transferred to a data sheet with a row for each single group of rats and columns for the different method parameters and for the results. If more than one of the included outcome measures were used in an article, they were prioritized according to how frequently they were used in the analysis as follows:

1. Size of ischemic lesion, most often evaluated with brain slicing and staining, but

in some cases with magnetic resonance imaging (MRI).

2. Histological evaluation of neuronal cell loss or survival, most often in the hippocampus.

3. Neurologic deficit score.

4. Other outcome measures.

In the case that more than one moment or location of damage evaluation was given for one or more groups of rats, and no summarized result were presented, the numbers most representative for the study's total result, or the most popularly evaluated site of injury, for example the CA1 region in the hippocampus, were selected.

### 4.9 Statistics

The choice of statistical methods is as complicated as it is important. Two questions that were frequently encountered and pondered upon when analyzing the results in this thesis were if the data could be considered normally distributed and how to handle outliers, which is especially relevant when performing steroid hormone RIAs.

In Paper 1,  $17\beta$ -estradiol concentrations of treatment groups from different sampling occasions were analyzed by analysis of variance (ANOVA) with Tukey's post-hoc test (Systat version 11, Systat Software, Inc. San Jose, California). P-values  $<0.05$  were considered significant.  $17\beta$ -estradiol concentrations were logarithmically transformed before ANOVA due to non-Gaussian distribution of the non-transformed data. Outlier status was tested ( $p < 0.005$ ) by Dixon's test [302].

In Paper 2, ratio scale variables were analyzed by Pearson's correlation analysis using the proportional damage in estrogen treated versus placebo treated groups (abbreviated E/P-ratio), weighted against the number of animals in each study. If for example the area of ischemic lesion was halved by the estrogen treatment, the E/P-ratio was 0.5, and if the area was exactly the same in the experimental groups, the E/P-ratio was 1.0. For "positive" outcome measures (for example cell count in hippocampus) the E/P-ratio was inversed to enable comparisons to studies using "negative" outcome measures (for example ischemic lesion area). The  $r^2$ -value shows the part of the overall variation in the data explained by a linear relation between the factors (Method Validator, version 1.1.9.0; Philippe Marquis, 1999, Metz, France).

In Paper 3, infarct sizes were analyzed using Kruskal-Wallis nonparametric one-way analysis of variance and multiple comparisons, using a computer program described earlier [303]. Differences with p-values  $<0.05$  were considered significant. The mean and standard error of the mean (SEM) were used to describe central tendency and variation respectively throughout the article.

In Paper 4,  $17\beta$ -estradiol concentrations and uterus weights of the experimental groups were compared to the ovariectomized control group's results by Welch's t-test. All calculations were subsequently Bonferroni adjusted so that the risk of type 1 errors with significance level of  $p < 0.05$  was 5 % within each administration regimen. The two groups with silastic capsules were compared in each time-point using the same methods. All calculations were performed using QuickCalcs (2005, Graphpad Software, Inc. CA, USA). Hormone concentrations and uterine weights were presented as mean $\pm$ SEM throughout. Pearson's linear correlation was used for comparing estrone sulfate concentrations with simultaneous  $17\beta$ -estradiol concentrations. Outlier status was tested ( $p < 0.05$ ) by Dixon's test [302].

In Paper 5, an a priori power analysis with an expected coefficient of variation (CV%) of 40 for lesion sizes, and an expected lesion size increase of 40 % in the high dose group was performed, showing that 20 rats in each group would give a power of 0.869. When data had been obtained, the effect of the  $17\beta$ -estradiol administration regimen on lesion size was analyzed by ANOVA. Also, although not directly addressing the main hypothesis, the effect of  $17\beta$ -estradiol administration regimen on functional tests at day 17 was analyzed by one-way ANOVA, and its effect in combination with the covariate time on body weight, food and water consumption and serum  $17\beta$ -estradiol concentrations were analyzed by two-way ANOVA. Tukey's post-hoc test was performed on all ANOVA analyses (Systat 11). Significant differences with p-values  $< 0.05$  were considered significant. Data were presented as mean $\pm$ SEM, except when presenting serum  $17\beta$ -estradiol concentrations. The reason for using median and interquartile range instead was that several outliers, despite re-runs of the analysis, would have made mean $\pm$ SEM unrepresentative of the group.

#### 4.10 Exclusions and protocol violations

In Paper 1, one animal was excluded from the native control group because of an anatomical anomaly that made vaginal smear impossible. Day 16 of the experiment five animals in the slow-release pellet groups were found to have large crusts under the fur on their bellies. Day 36 this was also found in one animal in the group receiving injections. These were excluded from the study from the day of discovering the crusts. One animal in the silastic capsule group died during blood sampling anesthesia on day 28 of the experiment. In all, 8/120 animals were excluded during the experiment. Four  $17\beta$ -estradiol concentrations from serum samples were considered as extreme outliers and were therefore excluded from the significance analysis.

In Paper 2, the meta-analysis protocol was followed without violations.

In Paper 3, one rat was excluded because of technical problems during surgery. All scanned brain slice images from three animals were lost in the analysis process. Measured concentrations of  $17\beta$ -estradiol in three serum samples were excluded due to technical problems.

In Paper 4, four rats in the pellet groups were excluded due to pulmonary edema or

cardiac tamponade. One rat in a silastic capsule group was excluded because the silastic capsule pushed out through the skin. All data from these animals were discarded and the animals replaced. One rat in long-term peroral group suddenly refused to eat Nutella after 1 week and was therefore excluded without replacement. Sixteen of the 745 samples contained extremely high  $17\beta$ -estradiol concentrations and were excluded as outliers.

In Paper 5, one animal in the vehicle group and one animal in the low dose group were excluded during MCAo due to excessive bleeding from the ICA and intracerebral hemorrhage, respectively.

## 5. Results and Discussion

### 5.1 Differences between administration methods

#### – Papers 1 and 4

The unexpected finding in the study by Theodorsson and Theodorsson in 2005 [1], that  $17\beta$ -estradiol was damaging in our rat model of focal cerebral ischemia, was the motivation for initiating a thorough characterization of the methods used in that experiment, one of these being the commercial slow-release pellets manufactured by IRA. The literature was scrutinized in order to find data on the performance of hormone administration methods in rats; however it could soon be concluded that this was a sparsely investigated field. This insight motivated a study aiming to compare serum levels produced by administering  $17\beta$ -estradiol through pellets versus subcutaneous injections. Despite some technical problems in the hormone analysis it could clearly be seen that the commercial pellets did not at all result in an even release of hormone, as stated by the company, but on the contrary needed several weeks to reach steady-state. A similar pattern was seen in the group receiving injections [304]. Although this provided some information about these two administration methods, more was wanted, and thus another experiment was planned.

In this next experiment, resulting in Paper 1 of the current thesis, a third method was added, namely a type of home-made silastic capsule. These capsules had been well-used in rat experiments of estrogens and cerebral ischemia, though studies actually assessing their performance were almost as scarce as in the case of the commercial slow-release pellets. Since there is an inevitable number of alternatives on how to assemble the capsules, we selected the type designed by Wise et al., that often had been used in experiments where estrogenic neuroprotection was reported [261]. Also, another difference in hormone administration between the protocol used in our lab and the studies by Wise et al. was addressed – the length of time between ovariectomy and hormone administration, also known as the wash-out period. Wise used no wash-out period, while two weeks had been used in our group, so two groups of animals in the study received the exact same silastic capsule, but with and without two weeks of wash out. In Paper 4, the same methods, with the addition of a novel peroral method, were tested in a short-term perspective.

The main findings of Papers 1 and 4 were that the tested home-made silastic capsules proved superior to the IRA pellets and subcutaneous injections in producing serum hormone concentrations that were, although steadily sinking, within the physiological range for at least four weeks. In concordance with the previous pellet evaluation [304], the expensive commercial pellets produced serum levels with an early, highly supraphysiological peak, that later substantially decreased. Ironically, the pellet that was supposed to last for 90 days dropped before the one supposed to last for 60 days. The injections resulted in fluctuating

and successively increasing concentrations with huge intra-group variations. Also, the injections induced daily surges of the hormone, very far from the relative stability of the silastic capsules. Another major drawback of the injection method was that it required daily handling of the animals, which was time-consuming and stressful for the rats. Besides the silastic capsule method, another delivery mode that showed great promise was the peroral regimen. It resulted in daily peaks just above the physiological spectrum, that however were far from the extremes produced by injections (Figure 9).

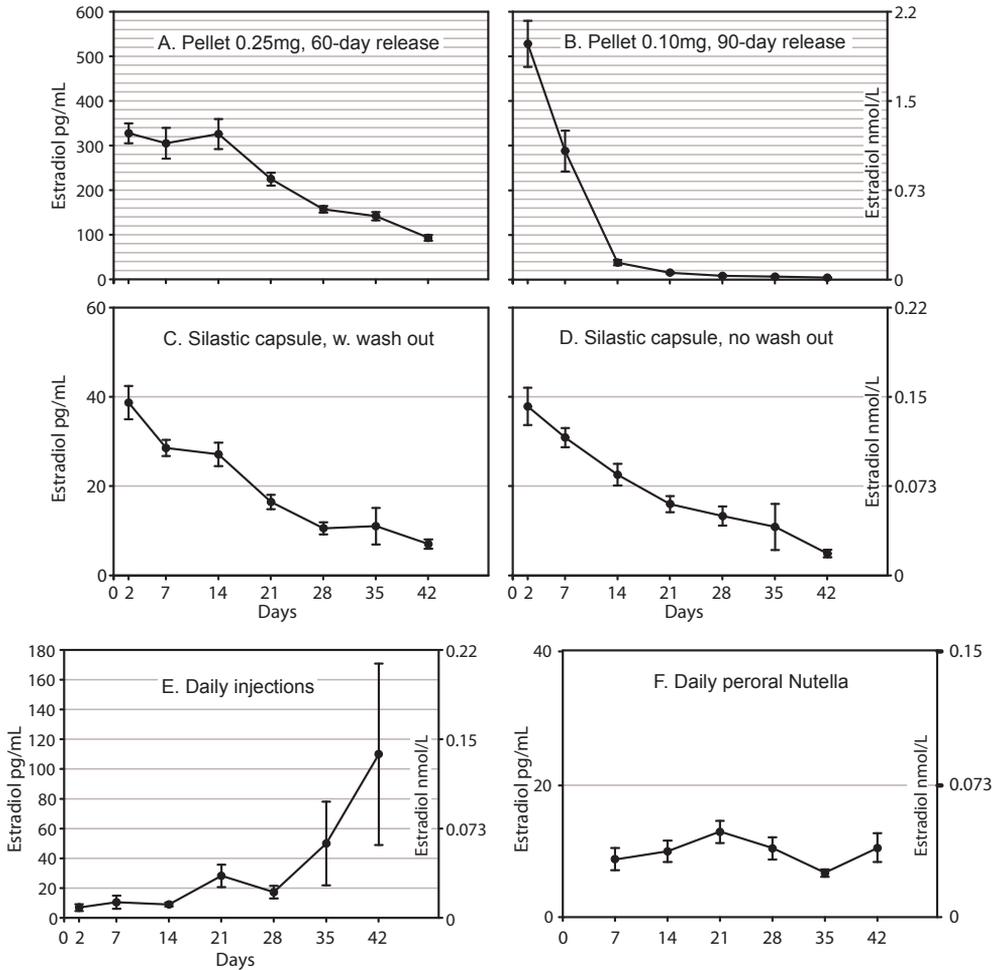


Figure 9. The most important results from Papers 1 and 4 were that the slow-release pellets (A and B) produced concentrations that were an order of magnitude above the physiological range (which is about 5-100 pg/mL), while the silastic capsules (C and D) and peroral method (F) produced predominantly physiological levels. The daily injections (E) caused extremely high daily spikes (not seen in the figure) and considerable intra-group variability. Results are plotted as mean $\pm$ SEM, n=15. The difference in Y-axis scale between the individual graphs should be noted. In all plots, there are grey horizontal lines every 20 pg/mL to facilitate comparison between the graphs [previously published in Papers 1 and 4].

These findings had several significant consequences. First, since the silastic capsules produced hormone concentrations that were within the physiological range, studies in which they have been used have a stronger biological relevance. The IRA pellets' supraphysiological hormone levels could of course still be of interest for animal models where high pharmacological concentrations are required, though the extreme changes in serum levels over time, without clear relation to the stated release-time, make them an unreliable tool. Injections seem to have more disadvantages than benefits for longer studies, though if short-time administration is desired, the method could be an option. If similarity to clinical peroral estrogen therapy is wanted, the Nutella® method is an attractive alternative. The results published in Papers 1 and 4 offer a possibility for researchers to choose between four well-characterized estrogen administration methods, each with clearly defined benefits and drawbacks (Table 1).

**Table 1. Benefits and drawbacks of different 17 $\beta$ -estradiol administration methods**

Method	Benefits	Drawbacks
Subcutaneous slow-release pellet	<ul style="list-style-type: none"> <li>• One single administration</li> <li>• Convenient to order</li> </ul>	<ul style="list-style-type: none"> <li>• Produces a high serum concentration peak the first days</li> <li>• High and unpredictable serum concentrations</li> <li>• Expensive</li> <li>• Requires anesthesia</li> </ul>
Subcutaneous silastic capsule	<ul style="list-style-type: none"> <li>• After the initial peak, it produces physiological serum concentrations for at least four weeks</li> <li>• One single administration</li> <li>• Cheap</li> </ul>	<ul style="list-style-type: none"> <li>• Produces a high serum concentration peak the first hours</li> <li>• Requires anesthesia</li> <li>• Must be assembled by the researcher soon before administration due to uncertain expiration date</li> </ul>
Subcutaneous injections	<ul style="list-style-type: none"> <li>• Uncomplicated</li> <li>• Require no anesthesia</li> <li>• Cheap</li> </ul>	<ul style="list-style-type: none"> <li>• Produce a very high serum concentration peak every day</li> <li>• Large intra-group variability</li> <li>• Require daily, stressful handling of the animals</li> </ul>
Peroral Nutella®	<ul style="list-style-type: none"> <li>• Produces physiological serum concentrations</li> <li>• Non-invasive</li> <li>• Mimics clinical treatments</li> <li>• Cheap</li> </ul>	<ul style="list-style-type: none"> <li>• Requires daily attention</li> <li>• Animals have to ingest Nutella®</li> </ul>

As aforementioned, the impact of a wash-out period was assessed in Papers 1 and 4. It was found that in terms of achieved serum  $17\beta$ -estradiol concentrations, such a convalescence period did not seem to have an effect, which was a somewhat unexpected finding. Theoretically, two weeks of hormone depletion could alter both the serum protein profile, including SHBG, and the enzymes responsible for the biological conversion of estrogens, which in turn could affect the serum  $17\beta$ -estradiol concentrations once the hormone had been administered. But since the serum concentrations were unaffected, a wash-out period may in this regard be omitted (Figure 9C and D).

### **5.2 The estrogen-stroke dichotomy may be explained by administration methods – Papers 2, 3 and 5**

The finding in Paper 1 (and later in Paper 4), that there were profound differences in performance between the four tested administration methods, encouraged us to further investigate the impact of the experimental methodology. To test the hypothesis that the divergent results concerning estrogens' effects on cerebral ischemia could be explained by methodological discrepancies, we planned to perform a meta-analysis and collected articles where estrogens' effects on stroke had been tested in rat models. An extensive literature search resulted in 66 articles that met our criteria of which 6 reported that estrogens increased the damage in cerebral ischemia. The articles had a relatively homogenous experimental setup, and thus constituted a suitable material for methodological comparisons and meta-analysis.

By analyzing the differences in results in relation to methods used it became clear that the administration methods were of fundamental significance. IRA pellets had been used in all studies where damaging effects of estrogen were reported [1, 41-45], despite that the pellets were adopted in a minority of the total number of studies (Table 2). A statistical calculation of the likeliness that this pattern would have occurred by pure chance was not included in the published article, but has been performed later. Using Chi<sup>2</sup> test, it was found that the administration methods affected the results with a p-value <0.001. Further, it seemed that a higher pellet dose increased the likeliness of more neurologic damage or less neuroprotection (Figure 10), a pattern that was also seen in studies where other administration methods had been used. None of the other methodological factors investigated – the method of inducing the ischemic brain lesions, the variables for measuring outcome, the measured serum concentrations of estrogens at the time of ischemia nor rat population attributes (sex, strain, age and diseases) – were found to contribute to the result dichotomy. This firmly suggested that the estrogen-stroke dichotomy in rat experimental studies was the result of differences in administration methods. The observed dose relation between estrogens and increased neurological damage in combination with the findings from Papers 1 and 4 suggested that the detrimental effects of the IRA pellets was a consequence of their initial supraphysiological serum hormone peak, being the main attribute by which this method was distinguished from the

other modes of administration.

Administration mode	Neuroprotection [number of studies]	No difference [number of studies]	Neurodamage [number of studies]
Injections	33	2	0
Pellets	12	3	6
Silastic capsules	16	0	0
Oral administration	1	0	0

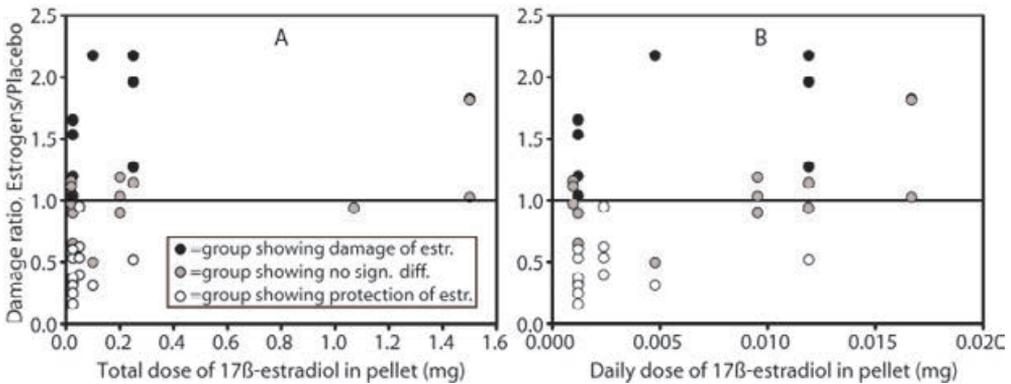


Figure 10. For each study where pellets were used an “E/P-ratio” between damage in estrogen treated against placebo treated rats was calculated. An E/P-ratio of 1 indicated that the estrogens had no impact on the ischemic damage. In the figures, E/P-ratio is plotted against the total dose of estrogens (A) and the daily dose of estrogens (B), respectively. Black, white and gray dots in the figure illustrate groups where statistically significant damaging effects, statistically significant protective effects or no statistically significant effects of estrogens were seen, respectively. Further, correlation analyses weighed in respect of the number of rats in each group were performed. The E/P-ratio correlated with total dose of estrogens with an  $r^2$ -value of 0.24 ( $r=0.49$ ), and with daily dose of estrogens with an  $r^2$ -value of 0.29 ( $r=0.54$ ) [previously published in Paper 2].

To put the finding that differences in administration methods seemed to be the main factor responsible for the dichotomous results to the test, the experiment leading to Paper 3 of the current thesis was set up. This experiment was designed to exactly mimic the earlier mentioned stroke study from our laboratory [1], except in regard of estrogen administration method. Instead of the earlier used IRA pellets, silastic capsules, assembled as the ones tested in Paper 1, were used (see Table 3 comparing the two studies). Three groups of rats received

17 $\beta$ -estradiol containing silastic capsules according to three different time schedules, and a fourth group received placebo capsules only. Three days after MCAo, the animals were sacrificed and infarct volumes measured. The major finding was that by changing administration method from IRA pellets to silastic capsules, the effect of 17 $\beta$ -estradiol was changed from increasing ischemic damage to protecting against it. This strengthened the hypothesis derived from Paper 2; that the administration regimen was a crucial factor to determine the effect of estrogens on stroke (Figure 11).

Methodological parameter	Theodorsson & Theodorsson 2005	Paper 3
Estrogen administration mode	Pellets from IRA	Home-made silastic capsules
Length of estrogen treatment	2 weeks	
Type of estrogen	17 $\beta$ -estradiol	
Method for induction of ischemic lesions	MCAo by microclip, reperfusion after 60 min	
Length of time between ischemia and damage evaluation	72 hours	
Type of outcome measure	TTC-staining and infarct size measurement	
Rat sex	Female	
Rat strain	Sprague-Dawley	
Rat age	3 months	
Rat diseases	None	
Miscellaneous	Same laboratory, surgeon and technical equipment used in both studies	
Effect of 17 $\beta$ -estradiol on focal cerebral ischemia	Neurodamage (p<0.001)	Neuroprotection (p=0.015)

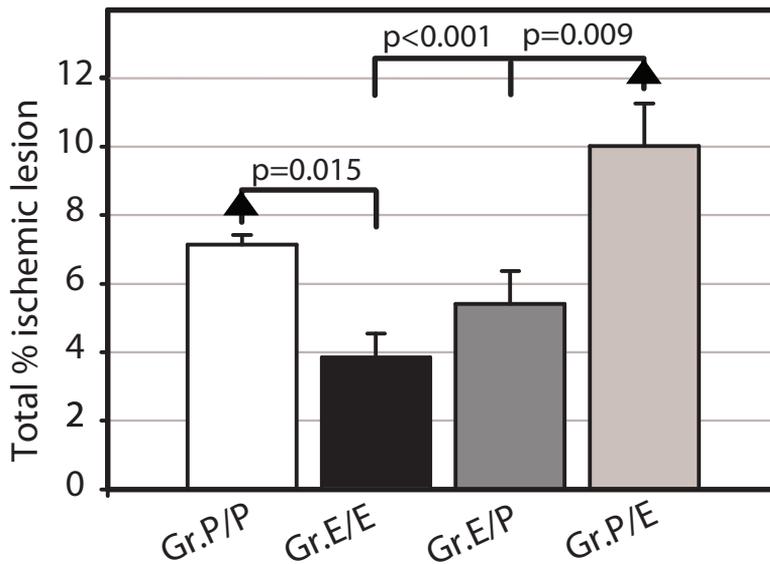


Figure 11. The mean ischemic lesion area calculated from all slices in each experimental group in Paper 3. The group receiving placebo capsules (Gr.P/P) had significantly larger lesions than the group that was estrogen treated throughout the entire study (Gr.E/E;  $p=0.015$ ). The two groups receiving  $17\beta$ -estradiol only before (Gr.E/P) or only after (Gr.P/E) the MCAo were not statistically different from the vehicle group [previously published in Paper 3].

However, we were not only interested in the impact of administration methods, but in the resulting administered dose and hormone serum concentrations per se, as reflected in the concept of hormesis. Even if the joint results from Papers 1-4 strongly suggested that the serum concentration was the main factor, this had not been proven in an experiment solely designed for this purpose. We therefore set up a study in which three rat groups, differing only in the  $17\beta$ -estradiol concentration contained in their silastic capsules, were inflicted focal cerebral ischemia. The hypothesis was that the vehicle group would sustain larger damage than the low dose group, receiving the capsules that earlier had been protective, but smaller than the high dose group, receiving capsules that were designed to mimic the slow-release pellets that earlier had been detrimental. Unfortunately, the intra-group variation was so large that any eventual inter-group differences were obscured, and no firm conclusions could thus be drawn (Figure 12).

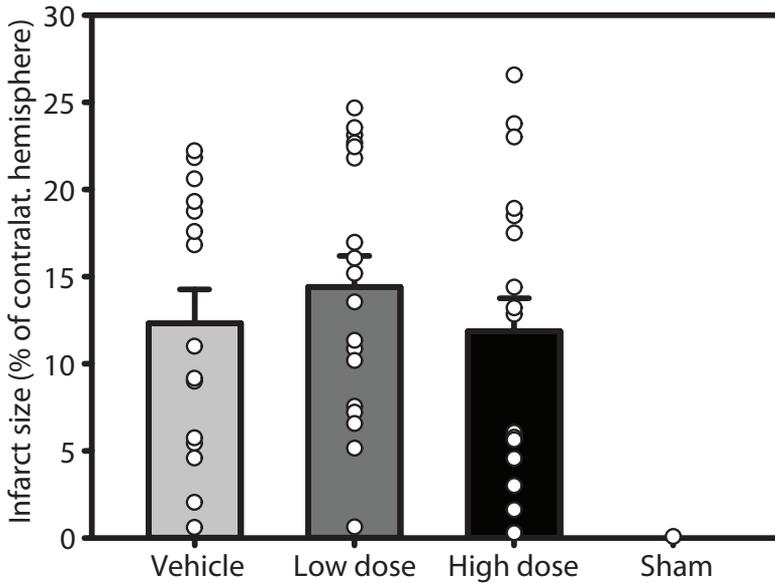


Figure 12. Infarct sizes ( $\pm$ SEM) in all groups in Paper 5. The white dots represent individual infarct sizes. There were no significant differences between the vehicle, low dose and high dose groups. A sham group, that did not sustain any ischemic damage, is also presented in the graph.

### 5.3 A dose perspective of mechanisms for estrogens' actions in stroke

If the mechanisms for estrogens protective of detrimental effects in stroke, described in the Introduction, are viewed from the perspective that low dose regimens are protective while high dose regimens are more likely to be damaging, an interesting pattern emerges. It seems that low-dose regimens, in comparison to placebo, are prone to decrease cerebral inflammation, which would be protective in ischemic stroke, while the opposite is found for prolonged high-dose regimens.

Of the studies above in which estrogens decreased cerebral inflammation, all but two adopted presumptive low-dose or short term  $17\beta$ -estradiol regimens, such as various intraperitoneal or subcutaneous injection schedules and low-dose silastic capsules, which are, according to Paper 2, in the dose range likely to induce protection against ischemic damage [46, 102, 105, 110, 111, 114, 118, 120, 121]. The remaining two of the abovementioned studies used the pellets from IRA [51, 119]. In one of these studies using pellets [119],  $17\beta$ -estradiol merely decreased the number of astrocytes and microglia without relation to stroke, which could be interpreted as a degenerative as well as an anti-inflammatory effect, and in the other pellet study, older rats given the same treatment showed increased cerebral inflammation [51]. Thus, of the studies reporting estrogen-induced decreases in animal brain inflammation, a majority has been performed with short-term or low-dose estrogens similar to regimens that previously have

been reported to decrease cerebral ischemic damage.

Most interestingly, in sharp contrast to the majority of studies reporting decreased cerebral inflammation, all but one [123] of the studies reporting increased cerebral inflammation from estrogens adopted administration regimens that are likely to exacerbate ischemic damage in rats [Papers 1 and 2]. The high-dose regimens used were slow-release pellets from IRA [45, 51, 125] and silastic capsules containing dissolved  $17\beta$ -estradiol in concentrations about 10 [124] to 250 [126] times higher than the highest dissolved silastic capsule  $17\beta$ -estradiol concentration that, to the best of our knowledge, has been reported to be neuroprotective [305]. The pro-inflammatory effects of estrogens have by the authors of these articles generally not been interpreted as resulting from the high hormone dose, but rather as synergistic effects of diabetes [45, 123] or old age [51, 125, 306]. However, a possible contribution of factors such as age and disease do not explain the striking dominance of high-dose regimens in these experiments, thereby suggesting that estrogens in supraphysiological concentrations are likely to have a higher propensity for increasing cerebral inflammation, in line with the hypothesis that high-dose estrogens increase damage from cerebral ischemia. It should also be mentioned that estrogens indeed have been reported to protect both diabetic and old animals in several studies, contradicting a clear relation between age, diabetes and neurodamaging effects of estrogens [39, 40, 307-309].

If we broaden our focus to include other organs than the brain, most experimental studies demonstrating hormetic phenomena of female sex hormones on inflammation suggest that low hormone concentrations are pro-inflammatory whereas high hormone concentrations are anti-inflammatory [242], such as the effects of estrogens on the pro-inflammatory cytokine IL-1 [247]. Even though these observations appear well in line with the understanding of the anti-inflammatory role of the high estrogen concentrations during pregnancy, aimed at avoiding abortion of the fetus, they contradict the pattern suggested by the abovementioned studies of estrogens' effects on inflammation in the brain. The discordant patterns concerning in which concentration-ranges estrogens are neuroprotective or neurotoxic could possibly result from organ differences or reflect discrepancies in the measured end-points.

The other abovementioned (in section 1.3.1) suggestions for estrogens protective effects, namely decreased apoptosis, growth factor regulation, vascular modulation and indirect decrease of oxidative stress by altering the anti-oxidative defense have all been demonstrated in experimental settings fitting the dose-concentration range pattern established for neuroprotection and are therefore likely candidates for being true protective mechanisms [310]. The direct anti-oxidative effect still needs to be demonstrated in relevant biological settings in estrogen concentration intervals known to be protective in whole-animal models, and this mechanism is especially difficult to assess because of the difficulties in separating genomic from non-genomic actions.

Hence, while several mechanisms seem likely to contribute to the neuroprotective effects of estrogens in lower concentration ranges, it seems that possibly the anti-inflammatory effect of estrogens turning pro-inflammatory in supraphysiological concentrations could explain the observation that estrogens have opposing effects in different concentrations (Figure 13). It should however be emphasized that assessing which mechanisms are true and false is a highly complex issue, and no firm conclusions can be drawn from a literature review of this kind.

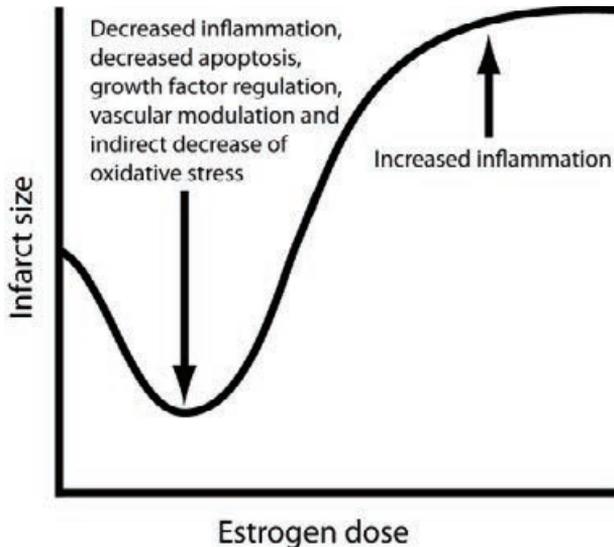


Figure 13. Hypothetical graph of estrogens' effect on stroke and its possible mechanisms. Whereas many mechanisms seem plausible to contribute to the protective effect, the only strong candidate for the damaging effects is the mechanism of increased inflammation.

#### 5.4 The possible relevance of the results to hormone replacement therapy

If the suggestion in the current thesis holds true – that the serum concentrations of 17 $\beta$ -estradiol resulting from the mode of administration, including dose and route, is of central importance for the negative or positive effects of estrogens – this has a bearing on the debate on use of HRT. Would it be possible to reduce the negative effects of HRT at the same time as conserving the amelioration of peri-menopausal symptoms by merely altering the administration regimen? It has been proposed in connection to WHI, that perhaps a different result on coronary heart disease and cerebrovascular events could be obtained by altering the HRT dosage [311].

It could be argued that there is an inherent problem in comparing the results from rat ischemia studies with the results from the human population studies in that there is a fundamental difference in outcome measures. While the human population studies used incidence of stroke, the animal studies, as in Paper 3

and 5, assessed stroke severity. Though, it merits attention that the diagnoses that are registered in the human population studies represent different grades of focal cerebral ischemia, from “transient ischemic attack” (TIA), via the formerly used expression “reversible ischemic neurologic deficit” (RIND) to “stroke” and “lethal stroke”, mainly separated by severity of symptoms. Given that these different grades of focal cerebral ischemia are on a scale of symptoms from almost none to death, it seems reasonable that an increase in severity would be interpreted as a change of incidence in the different categories. For example, increasing the neuronal damage of the ischemia with estrogens would make a bigger part of the ischemic events to be registered as serious or even fatal and vice versa. Thus an increase in severity would be interpreted as an increase in incidence. This aspect is illustrated by the results from the randomized controlled trial Heart and Estrogen/progestin Replacement Study (HERS), where the trend of increased incidence of fatal stroke (1.61; 0.73–3.55) seemed to be bigger than the increase of stroke in general (1.23; 0.89–1.70), while the total number of cerebrovascular insults (including TIA) was almost unchanged (1.09; 0.84–1.43) [312]. Similar results were seen in a randomized controlled trial by Viscoli et al. 2001, where an increase in fatal stroke (2.9; 0.9 to 9.0), but not in nonfatal stroke (1.0; 0.7 to 1.4), was seen [313]. Hence, it seems reasonable that the impact of estrogens on ischemic damage seen in rat studies, despite the fact that the outcome is only measured as severity, at least partly could reflect the same biologic phenomenon as seen in the human population studies where the outcome measure is incidence.

It should also be emphasized that a problem with extrapolating the experimental animal data to humans is that different administration methods were used. In the human studies cited above (section 1.3), the hormones were orally ingested, while practically all animal studies used parenteral regimens. When administering orally, the hormone passes the liver before entering the systemic circulation, where it to large extent is metabolized and also potentially affects coagulation and lipid metabolism. The reason for designing the oral regimen in Paper 4 was to be able to address this gap between clinical and animal studies in the future.

If the administration design, in analogy with the findings in rats, is a crucial factor in humans as well, it is probably only one piece of the puzzle concerning the relation between HRT and stroke. Although it seems to be the main factor causing the dichotomy in the rat experimental studies, too little is known to draw any firm conclusions regarding the human situation. The administration regimens tested in human population HRT trials were similar between studies, thus not substantiating a dose-dependent reason for the different results. Even though experimenting with HRT dosage regimens bear a certain degree of promise, other factors are likely to contribute to the HRT-stroke dichotomy.

## 5.5 Future perspectives

The obvious next step is to try to reduce the intra-group variability in lesion size obtained with the filament technique, to subsequently be able to address the hypothesis tested in Paper 5 anew. If we could show that the high doses are stably

damaging while low doses are protective, irrespective of the administration method in which the dose is packaged, it would be very interesting to pursue the mechanisms of this shift in effects. Even if mechanisms have been suggested for the damaging effects of estrogens, they have not been systematically evaluated in a robust model. To investigate mechanisms, various knock-out animals, such as ER knock-out mice, could be used, or the effect of different inflammatory inhibitors or triggers in combination with low and high estrogen doses could be tested. Mapping the mechanisms for not only the protective, but also the damaging, effects of estrogens in stroke could contribute to the development of safe HRT regimens or even stroke-protective drugs.

A concern when trying to extrapolate, if even with great caution, the results in the current thesis to the human situation is that the serum hormone levels needed to produce a damaging effect of estrogens in rats seems to be arguably much higher than those obtained by human HRT. We therefore see the importance of investigating whether different animals have different thresholds for the detrimental effects. In some of the studies mentioned above (sections 1.3.1.2 and 5.3), estrogens caused increased inflammation in old or diabetic animals to a greater extent than in young, healthy animals. Could it be that old animals with various diseases are less “estrogen tolerant”, and thus estrogens in these animals in a lower dose range switch from being protective to damaging? The eventual finding of such a phenomenon could partially bridge the gap between the animal models and the human situation.

### **5.6 Strengths and weaknesses of the thesis**

The main strength of the current thesis and the related research conducted in our laboratory during recent years is that there has been a serious effort to thoroughly investigate the used methods, and we have constantly worked on improving the study designs accordingly. Even if methodological research is frequently perceived as a necessary evil or even a waste of time, it has proven both fruitful and rewarding to us. The methodological studies have not only provided interesting insight regarding the effects of estrogens on stroke, but can also be of value to researchers working with other estrogen-related topics.

The thesis has several weaknesses, not least in Paper 3. The aim of that study was, as aforementioned (section 4.1), to exactly mimic an earlier experiment [1], except in terms of administration method. However, we overlooked the wash-out factor, which was omitted in Paper 3 even though it was included in the earlier publication. The reason for this was that the results from Paper 1 had made us abandon the use of wash-out periods, and it was not until after publication of Paper 3 that we realized that including a wash-out, to make the setting even more similar to the compared study, would have been optimal. Another mistake in Paper 3 was that blood samples were taken one day after MCAo from a total of 13 rats in two of the experimental groups, but not in the other 35 animals. Even if no such effect could be seen, anesthetizing an animal and drawing blood 24 h

after ischemia could theoretically affect the infarct size 48 h later. A smaller imperfection is how the lesion sizes were presented. Instead of presenting slice areas, it would have been much clearer to calculate the lesion volume, as performed in Paper 5. Further, no power calculation was performed before starting the experiment, however we emphasize in the article that the lack of significant differences between some of the groups are “non-findings”.

Blinding and randomization are two other issues that could have been handled with more scrutiny in Papers 1, 3 and 4. In none of these studies, the experimenters were blinded to the group allocations, except for the histological assessment in Paper 4. Also, even though the animals in Papers 1, 3 and 4 were randomized to the different groups, the rats were not handled in random order in Papers 1 and 4. Instead, the groups were handled one at the time (although overlapping). The reason for the consecutive handling of the groups was to minimize the risk of mixing the animals or contaminate one group with another. In Paper 5, however, the experimenter was blinded and the handling performed in random order.

The large intra-group variability in Paper 5, depriving it of statistical power and thereby probably contributing to the negative result, is of course a weakness of that study. We are currently working to refine the filament model by modifying three crucial parameters: A) awakening the animal during MCA occlusion or keeping it asleep, B) the size of the occluding filament and C) the length of the occlusion. If this had been done more carefully before initiating the study, more interesting results may have been obtained.

## 6. Conclusion

Starting with the unexpected finding that  $17\beta$ -estradiol when delivered in a high-dose pellet was detrimental in focal cerebral ischemia, we have subsequently gathered a considerable amount of data indicating that the dichotomy in the experimental estrogen-stroke field have resulted from the hormone administration methods used. The current thesis, including its review of a substantial amount of parallel or similar research, provides a prime example of the basic assumption that a thorough knowledge of the methods used is indispensable to correctly assess study data.

## 7. Errors

- In the Introduction of Paper 1, it is stated that the Women's Health initiative was an epidemiologic study, which it was not; it was a randomized controlled trial.
- In Figure 2 in Paper 1, the SI-unit scale on the right hand Y-axis is incorrect, and should be multiplied with a factor 3.
- In the Results in Paper 1, in the section "Group receiving 90-day release 0.10 mg pellet (Gr.P0.10)", it is written that "The animals in Gr.P0.10 had significantly lower serum concentrations of  $17\beta$ -oestradiol than those in **Gr.P0.** on days...". The second group (in bold) was supposed to be Gr.P0.25.
- In the figure legend to Figure 3 in Paper 1, it is stated that the light horizontal lines correspond to 20 pmol/L, which is incorrect. It should be 20 pg/mL instead.
- In Figure 3 in Paper 2 and Figure 2 of Paper 3, the slow-release pellet doses are wrong. Instead of "g", it should be "mg".

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