6

Brain Plasticity Following Ischemia: Effect of Estrogen and Other Cerebroprotective Drugs

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1. Introduction

Cytoprotection and brain regeneration are both potential future therapies in the treatment of cerebral ischemia, both based on animal research data. Since Kaas and colleagues first described the reorganization of the sensory cortex after peripherial nerve injury it has become clear that "we can no longer consider the injured brain as a normally wired brain with a missing puzzle piece" (Kaas et al., 1983 as cited in Nudo, 2006) and thus there has been intense research subsequently focused on understanding the regeneration processes following different brain injuries.

In rodent models, permanent and transient middle cerebral artery occlusions (MCAO) are the most relevant for representing human ischemic stroke. The lesioned brain areas in these experimental cases are well comparable to those found in humans. Global cerebral ischemia has been investigated using several models including (1) hypotension with bilateral carotid occlusion, or (2) four vessel (2 carotid arteries + 2 vertebral arteries) occlusion with normotension, (3) hypoxia, and (4) cardiac arrest models in rats or mice, or (5) the transient carotid occlusion in gerbil. These global brain ischemia models mimic severe hypotension, cardiac arrest, hypoxia, cardiac surgery, among other conditions in the human, however, while in rodent models the most vulnerable region is the hippocampus, in human both cortical and subcortical lesions are common. This is one reason why these models are difficult to translate into human studies in global cerebral ischemia, however, longer ischemic periods in rodents also result in patchy cortical and subcortical damage together with the hippocampal damage (Back et al., 2004; Erdő and Hossmann, 2007; Wappler et al.,

2009 and 2010). Though, these models are useful to investigate the pathophyshiological mechanisms of brain ischemia, but it is important to note that in human stroke patients there are usually several other factors to consider, such as obesity, hypertension, diabetes, age and medications a patient may be using (Li and Carmichael, 2006; Popa-Wagner et al., 2007; Wappler et al., 2010) which may also determine the size of the lesion together with the regenerative potential of the tissue. This emphasizes the importance of experiments that utilize disease model and not just young, healthy male subjects to investigate brain injury, which will hopefully bring us closer to an understanding of the complex clinical conditions that result in stroke (Nudo, 2007; Popa-Wagner et al., 2007; Wappler et al., 2010). In addition to understanding the process of ischemic brain injury, examining the potential effects of the drugs that afford protection against ischemia is just as crucial under different conditions.

In this chapter we present an overview of the studies describing the regenerative potential of the brain due to ischemic damage. Furthermore, we discuss drugs that increase brain plasticity after ischemic insult in animal models, focusing on estrogen. In addition, we describe a brief study examining acute 17β-estradiol treatment on synaptic plasticity in the brain with a short (4 days) and a long term (25 days) follow up in young (4 months old) and old (18 months old) gerbils after global cerebral ischemia. The aim of this study was to investigate whether changes in synaptic density can be maintained after a longer period of single dose estrogen treatment, as we have demonstrated an early induction of neuronal plasticity using this model (Wappler et al., 2011b). Maintenance of synaptic density may be an important factor underlying the previously described better functional outcome using this model that was investigated up to the 2nd week after brain ischemia (Wappler et al., 2010). In our current study, we examined two different age groups because synaptic reorganization is known to decrease with advancing age (Kim and Jones, 2010). Our results can help elucidate how synaptic reorganization progresses in the brain after global cerebral ischemia due to a single treatment of a cerebroprotective drug and how brain plasticity is influenced by age.

2. Brain regeneration after cerebral ischemia

Cerebral plasticity is the ability of the brain to change its structure and function during maturation, learning, environmental challenges or pathology (Di Filippo et al., 2008; Lledo et al., 2006). The exact mechanism of spontaneous brain regeneration after brain ischemia is not fully understood; however, there is a remarkable number of publications that describe several mechanisms participating in this process.

Here we discuss the following features in brain regeneration: 1. neural plasticity 2. vascular plasticity and 3. glial plasticity.

2.1 Neuronal plasticity

For almost one hundred years neuroscientists have believed that the adult primate brain, and therefore the human brain, is structurally stable and does not form new synapses or grow new cells (Gould et al., 1999). It is clear by now that certain brain regions generate new cells, and that the continuous “rewiring“ of the brain is an important physiological function.

Cortical interneurons but not pyramidal cells have been described to have intense arborization as axonal sprouting, dendritic growth and branching under physiological conditions (Lee et al., 2006) throughout adulthood. The "baseline" cerebral plasticity; however, is much more limited in the mature brain, compared to the developing brain, where high activity takes place. This phenomenon is generated by those structural and functional "brakes", such as myelin, and several neuromodulators, that actively suppress plasticity in the adulthood (Bavelier et al., 2010).

Following cerebral ischemia, or other types of brain insult; however, neuronal plasticity is reactivated in the surviving cells in order to compensate for cell death and to preserve functionality of the damaged but not dead areas (Blizzard et al. 2011; Carmichael, 2006). The possible mechanisms of neural plasticity include dendritic reorganization, axonal sprouting, and activation of endogenous pluripotent cells that can differentiate into neurons. While it has been shown that interneurons undergo structural remodeling in post-traumatic cortical lesions, signs of neural plasticity have not been detected in pyramidal neurons (Blizzard et al., 2011).

2.1.1 Dendritic, axonal and synaptic plasticity

Early studies demonstrated that following entorhinal lesion, as the result of neuronal projections from the contralateral hippocampus, new synapses are formed in the damaged cortex (Lynch et al., 1973). In addition, several subsequent studies have shown dendritic and axonal reorganization after experimental brain ischemia with dynamic changes of synaptic density in the injured brain region (Benowitz and Carmichael, 2010; Brown et al., 2010; Lu et al., 2004; Mostany et al., 2010; Scheff et al., 2005; Sulkowski et al., 2006; Takatsuru et al., 2009; etc.). The most active neuronal regeneration occurs up to 2-3 weeks after brain injury (Blizzard et al. 2011; Jones and Schallert, 1992), which provides a wide therapeutic window in cerebral ischemia.

Cerebral ischemia induces axonal sprouting within the peri -infarct zone and contralateral side. This post-ischemic axonal sprouting establishes new neuronal connection pattern for the damaged brain areas (Carmichael, 2003). Axonal sprouting after different central nervous system injuries can be detected by using growth-associated protein-43 (GAP-43) as it is a marker of regeneration in the adulthood (Benowitz and Routtenberg, 1987; Benowitz and Perrone-Bizzozero, 1991; Simon et al., 2001; Wappler et al., 2011b). During brain development GAP-43 is highly associated with the elongating axons (Benowitz and Routtenberg, 1987; Benowitz and Perrone-Bizzozero, 1991), which becomes less concentrated proximal to the cell soma while the axon is growing, but stays in the growth cone (Benowitz and Perrone-Bizzozero, 1991). GAP-43 is also thought to be involved in neurotransmitter release. The highest GAP-43 immunoreactivity was detected on the 4th postnatal day, when the most active synaptogenesis takes place. This is followed by a rapid decrease in the immunoreactivity with only a few brain regions expressing GAP-43 later in the adulthood. These regions are the cerebral cortex, the hippocampus, the hypothalamus, the amygdala, the striatum, the medial substantia nigra, raphe nuclei, locus coeruleus, olfactory bulb, olfactory tubercule, preoptic area, and stria terminalis (Benowitz and Perrone-Bizzozero, 1991; Yao et al., 1993). Each of these regions has a different level of constant reorganization. In the hippocampus, this reorganization is related to synaptic remodeling during memory formation (Benowitz and Perrone-Bizzozero, 1991; Holahan et

al., 2007). Astrocytes and perivascular extracellular matrix are involved in guiding axonal growth and provide a scaffolding surface for this growth (Nedergaard and Dirnagl, 2005).

Shortly after focal cerebral ischemia many neurons in the penumbra lose their dendritic spines in an attempt to survive (Brown et al., 2008; Benowitz and Carmichael, 2010). Nevertheless up to two weeks after stroke dendritic density and turnover increases in the peri-infarct cortex and also in the contralateral hemisphere, which plays a major role in brain regeneration (Brown et al., 2010; Mostany et al., 2010; Takatsuru et al., 2009). In addition, following cardiac arrest decreased microtubule-associated protein 2 (MAP-2) expression was detected in the rat reflecting lower dendritic density (Sulkowski et al., 2006). Recent studies have shown dendritic arbor shortening in the ischemic penumbra in the first weeks following stroke with further loss of dendritic branches after the first month in cortical pyramidal cells (Mostany and Portera-Cailliau, 2011). However, dendritic changes in the basilar tree of these cells or in other neuronal cell types in the cortex could not be ruled out. In contrast, enhanced dendritic arborization has been described in cortical pyramidal neurons following photothrombotic brain ischemia (Brown et al., 2010). Thus, the changes in dendrite vary among different cerebral ischemia models.

In different central nervous system injuries synaptogenesis (formation of new synapses) is critical because new connections restore the cell communication and signaling. Following injury, surviving neurons have been described to form new synapses to compensate for the lost contact surfaces even if pre-traumatic synapse number is not achieved in the traumatized area (Lu et al., 2004; Scheff et al., 2005). Therefore examining synaptic density is a widely used technique to track neuronal plasticity following brain lesions. Both synaptophysin (SYP) and synapsin-I are widely used synaptic markers to assess synaptic density. The vesicular transmembrane protein, SYP, is expressed in the presynaptic terminal and its expression seems to be dispensable in neurotransmission (Becher et al., 1996; Eshkind and Leube, 1995; McMahon et al., 1996) but may be involved in fine-tuning of synaptic activity and in vesicle biogenesis (Becher et al., 1996; Janz and Sudhof, 1998). Synapsin-I is a phosphoprotein located on the small synaptic vesicles in the presynaptic terminal (De Camilli et al., 1983; Schiebler et al., 1986), and participates in regulating plasticity (Roshal et al., 1993; Wei et al., 2011). Following axonal sprouting and dendritic reorganization, sometimes just 21 days after the ischemia, synaptic density increases suggesting the development of mature synapses (Stroemer et al., 1995 as cited in Carmichael, 2003). In addition it has been shown in a rodent model that synaptic density steadily decreased up to one week following global cerebral ischemia (Sulkowski et al., 2006).

2.1.2 Endogenous neurogenesis

While it was not believed that the adult human brain was able to form new neurons, Altman and Das provided the first evidence that neurogenesis occurred in the mature rodent brain (Altman and Das 1965 and 1967). These data opened a new chapter in neuroscience research. Besides the physiological functions, such as memory formation and learning, endogenous neurogenesis has become a major focus of research in different brain lesions (Gao et al., 2009; Kokaia and Lindvall, 2003; Shen et al., 2010; etc.) and their potential therapies (Kim et al., 2009; Leker et al., 2009; Xiong et al., 2011). The majority of the endogenous cerebral stem cells are located in the subventricular zone and hippocampal

subgranular zone and can generate either neuronal or glial cells (Zhao et al., 2008) in the lesion site using signals from the damaged cells for their activation and migration released.

These pluripotent cells express certain proteins that are typically present during brain development, and therefore are useful markers to track neurogenesis in adulthood following brain injury, such as ischemia. One of the markers is nestin, an intermediate filament protein, which is expressed in the astrocytes and radial glia cells in the developing brain and disappears after the 11th postnatal day in the rat (Kalman and Ajtai, 2001). Although previous data suggest that nestin immunopositivity in the adult bran is associated with immature cells that are involved in neuogenesis (von Bohlen und Halbach, 2007; Yue et al. 2006), it also a marker of reactive gliosis following various brain lesions (see e.g. Duggal et al. 1997). It also have been reported that following focal ischemia in the rat nestin positive cells from the ipsilateral subventricular zone can differentiate into glial cells (Holmin et al., 1997; Nakagomi et al., 2009; Shen et al., 2010). Therefore, in the adult brain, nestin expression recurs in both proliferating cells and in reactive astrocytes.

2.2 Vascular plasticity

Vascular plasticity includes processes such as angiogenesis and arteriogenesis. Angiogenesis is related to hypoxia and results in new capillaries from the pre-existing vessels, whereas arteriogenesis is induced most importantly by increased shear stress that results in newly formed blood vessels (Heil and Sharper, 2004; Heil et al., 2006; Schierling et al., 2009; Xiong et al., 2010); however, the differences in the cause and the result is usually are not this clear cut. Angiogenesis has a major role in brain regeneration after ischemia as increased blood supply directly enhances cell survival and regenerative processes (Font et al., 2010). Blood vessels not only provide metabolic support but also participate in neurogenesis by leading progenitor cells to the site of injury (Jin et al., 2002; Kojima et al., 2010 as cited in Font et al., 2010; Sun et al., 2010; Udo et al., 2008; Xiong et al., 2010; Yang et al., 2010).There is extensive evidence that neovascularization (both angiogenesis and arteriogenesis) is induced following acute (del Zoppo and Mabuchi, 2003; Issa et al., 2005) and chronic (Busch et al., 2003; Wappler et al., 2011a) brain ischemia.

Hypoxia induced hypoxia-inducible factor-1 (HIF-1) and vascular endothelial growth factor (VEGF) are the most common angiogenesis stimulators (Busch et al., 2003; Carmeliet and Collen, 1997; Liu et al., 1995; Levy et al., 1995) and are involved in capillary sprouting rather than in larger collateral vessel remodeling (Busch et al., 2003; Carmeliet and Collen, 1997) because they act on endothelial cells without inducing smooth muscle proliferation (Busch et al., 2003). In addition, several other factors, such as fibroblast growth factor (FGF) (Issa et al., 2005), angiopoetins (Lin et al., 2000), transforming growth factor β (TGFβ) (Haggani et al., 2005; Krupinski et al., 1996), platelet derived growth factor (PDGF) (Issa et al., 2005; Krupinski et al., 1997), tissue-type plasminogen activator (Carmeliet and Collen, 1997), etc. are just as critical in new vessel formation after brain injury. Most of these molecules have separate effects on cerebroprotection and regeneration, such as the TGFβ-s (Vincze et al., 2010). In addition, nitric oxide derived from endothelial nitric oxide synthetase (eNOS) also induces endothelial cell proliferation and migration, smooth muscle cell differentiation, and other angiogenic processes, where ischemia and shear stress are triggering mechanisms (Amano et al., 2003; Cui et al., 2009; Murohara et al., 1998; Papapetropoulos et al., 1997; Prior et al., 2003; Rudic et al., 1998). Angiogenesis inhibitors, such as endostatin, angiostatin,

thrombospondin-1 and thrombospondin-2 have also been detected following brain ischemia (Issa et al., 2005), which provide another avenue for therapeutic interventions.

In addition to these growth factors extracellular matrix proteins, such as laminin and dystroglycan complex (DGC) proteins, are also involved in vascular remodeling in the brain (Wappler et al., 2011a). The DGC proteins may have an important function in signal transduction connecting the extracellular signals and laminin itself with a wide variety of intracellular proteins, such as nitric oxide synthase, ion channels, kinases, and actin (Culligan and Ohlenieck, 2002; Wappler et al., 2011a). Thus, certain DGC proteins make good immunohistochemical markers of vascular reorganization (Wappler et al., 2011a) in addition to the more frequently used laminin.

Neuroregenerative agents that increase angiogenesis, such as estrogen (Ardelt et al., 2005), have been described to improve functional outcome in models of cerebral ischemia (Hermann and Zechariah, 2009; Goldstein, 2009). These experimental data correlate with clinical data where higher microvessel density in the brain after ischemia was accompanied by shorter recovery time and longer survival (Christoforidis et al., 2005; Font et al., 2010; Krupinski et al., 1994; Navarro-Sobrino et al., 2011; Wei et al., 2001).

2.3 Glial plasticity

Glial cells in the brain include astrocytes, microcytes, and oligodendrocytes, of which astrocytes are the most numerous. In the last decade glial cells have been recognized not just for structural but for metabolic and for throphic support. By secreting nerve growth factor [NGF], basic fibroblast growth factor [bFGF], transforming growth factor β [TFG-β], platelet-derived growth factor [PDGF], brain-derived neurotrophic factor [BDNF], ciliary neurotrophic factor, Neuropilin-1, vascular endothelial growth factor [VEGF], etc., they modulate the function of neurons and other cell types. Glial cells are active participants of synaptic interactions and higher level of cerebral function; and key elements of cerebral blood flow regulation (Araque and Navarrete, 2010; Attwell et al., 2010; Iadecola and Nedergaard, 2007; Metea and Newman, 2006; Nedergaard and Dirnagl, 2005). Glial cells also play a key role in regulating neuronal survival and regeneration by regulating the extracellular ion homeostasis, supporting other cells` energy metabolism, reducing glutamate toxicity, promoting neurogenesis, synaptogenesis and angiogenesis, activating endothelial cells, disrupting blood brain barrier (BBB), increasing inflammation, etc. (Himeda et al., 2007; Nedergaard and Dirnagl, 2005; Trendelenburg and Dirnagl, 2005). Generating new astrocytes is also an important feature in brain regeneration that has been mentioned previously in this chapter.

The formation of glial scar and its beneficial and non-beneficial properties are also of great interest when investigating astrocytic reaction following focal brain ischemia. Unlike other tissues where injury repair results in a fibrous scar, brain injury is followed by a special scar formed by the activated astorocytes and the extracellular matrix molecules of proteoglycans. These include heparan sulfate, dermatan keratan sulfate, sulfate proteoglycans, and chondroitin sulfate, which are released by reactive astrocytes to compose a barrier of axonal growth. The role glial scar formation following brain ischemia is still unknown and intense research is ongoing to understand if it is harmful or supportive (Rolls et al., 2009; Silver and Miller, 2004). Besides its support on the injured tissue (Rolls et al., 2009), its inhibitory effect

on axonal growth is equally important (Cole and McCabe, 1991; Filder et al., 1999; Katoh-Semba et al., 1995; Rudge and Silver, 1990; Smith-Thomas et al., 1994; Snow et al., 1990). Nevertheless, sulphated proteoglycans have also been described as supporting axonal growth (Hikino et al., 2003; Nakanishi et al., 2006), making glial cell based therapeutic strategies more difficult to design. On In contrast, myelin-associated inhibitors from oligodendrocytes and myelin debris, namely myelin-associated glycoprotein (MAG), Nogo-A and oligodendrocyte myelin glycoprotein (OMgp) have clearly inhibitory effect on neurite outgrowth (for review see Yiu and He, 2006) and are under investigation because blocking their function results in enhanced brain regeneration.

3. Inducing cerebral plasticity following brain ischemia

A therapeutic window for drugs that increase neural plasticity is wider than those that target cytoprotection following cerebral ischemia (Zhang and Chopp, 2009) giving hope for improved functional outcomes in more stroke patients. A cerebroprotective drug can increase synaptic density in several ways. Cytoprotection, a process where cells can utilize more energy to maintain features that are not necessary in cell survival, can contribute to increased neuronal survival and therefore plasticity in the injured area. Presumably for the same reason increased oxygen and metabolic support improve cellular plasticity after different ischemic events in the brain as already mentioned above.

Whether anti-apoptotic genes are able to induce neuronal plasticity by themselves other than by improving metabolic status is important to understand the pathophysiology of brain ischemia. In order to investigate this question we used Bcl-Xl or Bcl-2 gene construct transfections in an in vitro hypoxia model and we observed increased expression of synaptophysin-I and nestin mRNAs and proteins under normoxic conditions. Following hypoxia only nestin expression was significantly different from the untreated hypoxic group (Gal et al., 2009). These data indicate increased that anti-apoptotic gene expression itself can contribute to the amelioration of brain plasticity and its effect might be modified under different stress conditions. Several drugs that are known to be cytoprotective against cerebral ischemia, such as (-)-D-Deprenyl (Simon et al, 2001), and 17β-estradiol (Wappler et al., 2011), also participate in brain regeneration. Although both have anti-apoptotic effect, (-)-D-Deprenyl increases GAP-43 expression whereas 17β-estradiol treatment does not, suggesting that similar pathways may mediate enhanced regeneration through different intracellular signaling (Simon et al, 2001; Szilagyi et al., 2009; Wappler et al., 2011*)* both *in vitro* and *in vivo*. This is supported by btudies on other cerebroprotective drugs, such as erythropoietin (EPO) (Iwai et al., 2010), statins (Céspedes-Rubio et al., 2010), amphetamine (Liu et al., 2011), melatonin (Chen et al., 2009; González-Burgos et al., 2007), and different spices (Kannappan et al., 2011), where different ways of imporoved brain plasticity was described.

3.1 Estrogen

3.1.1 Estrogen in the brain, estrogen receptors

Corpechot and colleges described the first time that the cerebral sex steroid concentration is much higher than the circulating estrogen level both in men and women (Corpechot et al.,

1981). Subsequently, estrogen synthesis (Le Gascone et al., 1987) and the essential enzymes (Hojo et al., 2004) involved were detected in the brain.

The majority of the investigated intracellular effects of estrogen are related to two estrogen receptors (ERs) in the brain, ER-α and ER-β. Both of these receptors are expressed in the central nervous system; however, their distribution show different pattern. While ER-α is highly expressed in the hippocampus, hypothalamus, and preoptic area accompanied by a low expression in the cortex, ER-β is densely expressed in the cortex together with a high receptor density in the hippocampus, amygdala, cerebellum, etc. (Brann et al., 2011; Merchenthaler et al., 2003; Shughrue et al., 1997, Shughrue and Merchenthaler, 2000). Both of these ERs form homo- or hetero dimers after binding an estrogen molecule, such as estrone, estriol, or the most effective 17β-estradiol (E2). These dimers can bind to the estrogen responsive elements of the DNA and regulate the expression of several genes, such as bcl-2, IGF-1 (insulin like growth factor-1), NGF, (BDNF (McKenna and O'Malley, 2002; Merchenthaler et al., 2003; Nilsson et al., 2001; Sharma and Mehra, 2008). However, besides this "classical" signaling pathway, which requires hours to days to take place, estrogen can induce rapid changes via its non-genomic pathways. These non-genomic responses are mediated through extranuclear ERs, and occur within minutes of estrogen exposure through activation of several signaling cascades, such as phosphatidylinositol-3-kinase (PI3K), extracellular signal regulated kinase (ERK), mitogen-activated protein kinase (MAPK) or protein kinase C (PKC) (Bourque et al., 2009; Brann et al., 2011; Koszegi et al., 2011; Lebesgue et al., 2009; Rebas et al., 2005). Extranuclear receptors have been detected in the cytoplasm of the cell body, but also in the dendrites and axons of the neurons, while ER immunoreactivity was also seen in the organelle membranes, and synaptic vesicles (Milner et el., 2005). In addition, other brain cells, such as glial cells, have also been shown to express ERs (Milner et al., 2005; Woolley, 2007).

A third estrogen receptor, the G-protein-coupled ER (GPR30), has also been detected in the brain (Funakoshi et al., 2006); however, limited data is available regarding its role under physiological and pathological conditions. One of its reported functions in the hippocampus is to increase synaptic transmission (Filardo et al., 2002; Lebesgue et al., 2009). This receptor is more likely to be associated with the ERK/CREB intracellular signaling pathway (Lebesgue et al., 2009), and presumably activates other intracellular signaling cascades as well. GPR30 protein expression was described in the neuronal plasma membrane and endoplasmic reticulum in several brain regions, such as the hippocampus (Funakoshi et al., 2006; Matsuda et al., 2008).

There is strong evidence that the three different estrogen receptors can crosstalk, for example regulating gene expression through ERK and Src signaling via transcription factor, and histone phosphorylation (Brann et al., 2011; Madak-Erdogan et al., 2008). GPR30 pathway also can crosstalk with other extranuclear pathways through Akt activation (Lebesgue et al., 2009).

The effect of age on ERs is worth to mention here as the incidence of cerebral ischemia is higher in the elderly and the old, which can modify the effect of estrogen therapy. Both ER-α and ER-β expressing cell number decreased significantly in the hippocampus of the aged rats; however, optical density of immunoreactivity per cell showed a significant increase for both ER-α and ER-β immunoreactivity in the CA1 neurons, whereas in CA3 neurons, it was

significantly reduced (Mehra et al., 2005). Increased expression of ERs per cell is supposedly a compensatory phenomenon. ER-β immunoreactivity was, however, found decreased in the CA1 dendritic synapses in old female rats in another study (Waters et al., 2011).

3.1.2 Estrogen: Afforded protection and plasticity following brain ischemia

Estrogens are known to increase synaptic density in the intact brain (McEwen, 2002; Merchenthaler et al., 2003; Rune et al., 2006; Sá et al., 2009; Sharma et al., 2007; Woolley, 2007) even following administration of a single dose of this hormone (Sá et al., 2009; Wappler et al., 2011b). Even during oestrus cycle there is an intense fluctuation in dendritic density in rodents. Furthermore, ovariectomy or menopause itself results in a significant decrease of synaptic and dendritic density (Ojo et al., 2011; Woolley and McEwen, 1992). Data on estrogen effect also suggest that it acts directly at synapses by activating second messenger signaling, resulting in a rapid altering in neuronal excitability, synaptic transmission, and/or synaptic plasticity (Woolley, 2007). There is; however, limited data on neuronal plasticity following brain ischemia (Wappler et al., 2011b).

Several studies have shown that E2 therapy is neuroprotective in cerebral ischemia. Estrogen increases the number of surviving cells following ischemia (Liu et al., 2009; Merchenthaler et al., 2003; Platha et al., 2004; Wappler et al., 2010), reduces excitotoxicity (Connell et el., 2007; Herson et al., 2009; Weaver et al., 1997), inflammation (Herson et al., 2009 ; Stein, 2001; Suzuki et al., 2007), moderates blood–brain barrier dysfunction (Liu et al., 2005), is antioxidant (Connell et el., 2007), increases cerebral blood flow (Herson et al., 2009; Hurn et al., 1995; Pelligrino et al., 1998), reduces spontaneous postischemic hyperthermia (Platha et al., 2004), etc. Cerebral ischemia studies in ER-α and ER-β KO mice models, and pharmacological receptor inhibition have shown that ER-α is the primary mediator of neuroprotection. (Brann et al., 2011; Dubal et al., 2001; Liu et al., 2009; Merchenthaler et al., 2003; Miller et al., 2005). Both genomic and non-genimic effects seem to be involved in estrogen afforded neuroprotection (Merchenthaler et al., 2003). Selective GPR30 agonists have also been found neuroprotective in *in vitro* and *in vivo* models of brain ischemia (Gingerich et al., 2010; Lebesque et al., 2010; Zhang et al., 2010), however, its specific role in the pathophysiology of ischemic attack is still unknown.

Genomic effects of estrogen includes the inhibition of apoptosis (through bcl-2, bax, caspase-3); the diminution of inflammation (e.g. through tumor necrosis factor-α; interleukin 1, and 6); the induction of growth factor, structural protein, and neuropeptide expression; etc. (Merchenthaler et al., 2003). High dose, acute estrogen treatment in global cerebral ischemia also induces cerebral plasticity by increasing synapsin-I and nestin gene expression in gerbils as we described previously (Wappler et al., 2011b). GAP-43 expression was however not elevated further due to the treatment compared to the already increased level after brain ischemia in our model.

Most of the data on estrogen`s effect in brain ischemia were observed following chronic rather than acute treatment in rodents, which is a postmenopausal estrogen supplementation model as opposed to a model of acute therapy. There have also been a small number of studies that used older, or diseased animals, or females, especially investigating long term outcome (Wappler et al., 2010).

4. Estrogen modulates synaptic density age, and subregion dependently in the gerbil hippocampus after global brain ischemia

Our previous reports have demonstrated protective effects of E2 pre-treatment in gerbils following ischemia by increased cell survival, memory function and attention (Wappler et al., 2010). We also showed that increased cerebral plasticity takes place 4 days after the ischemia in the same model (Wappler et al., 2011b). Therefore, we hypothesized that E2 pre-treatment increase the hippocampal synaptic plasticity both in shorter (4 days) and longer (25 days) time points in gerbils at different ages.

4.1 Materials and methods

4.1.1 Animals

Ovariectomized gerbils of 4 (young), and 18 (old) months of age were used in our experiments. The animals were housed in an air-conditioned room at 22±1 ◦C with a 12 h light/dark cycle, and had free access to water and food. All the procedures on animals were approved by the Animal Examination Ethical Council of the Animal Protection Advisory Board at the Semmelweis University, Budapest, Hungary.

4.1.2 Surgery and 17β-estradiol treatment

The gerbils were anaesthetized with halothane (induction: 4%, maintaining: 1.5-2.5%) in a 30% O_2/70% N_2O mixture, breathing spontaneously via a face mask. Bilateral ovariectomy, and 10 min bilateral carotid occlusion or sham neck surgery were performed as previously described (Wappler et al. 2010). Briefly, bilateral ovariectomy was performed through lateral incisions in each animal. Two weeks later transient bilateral carotid artery occlusion (10 min) was established through a midline cervical incision using atraumatic aneurysm clips (Codman, Johnson and Johnson, Le Locle, NE, Switzerland). The neck tissue was reunited in two layers with non-absorbable, 4.0 silk sutures (Ethicon, Johnson and Johnson). Sham surgery consisted of the midline cervical incision and carotid preparation followed by 10 min period, after which the incision was closed. Thirty minutes prior to surgery, estradiol treated group wasgiven 17β-estradiol (Sigma Chemical Co. St Louis, MO, USA) 0.4 ml/100 g (4 mg/kg) body weight. On the other hand, sham-operated and untreated ischemic animals were injected vehicle solution (50% alcohol in normal saline) in a dose of 0.4 ml/100 g body weight intraperitoneally.

4.1.3 Immunohistochemistry

On the post-operative day (POD) 4 or 25 (n=5 in each groups) animals were sacrificed under deep halothane anesthesia, and brains were isolated and immersion fixed first in 10% buffered paraformaldehyde for 2 days, then in 4% buffered paraformaldehyde for another 5 days. The brain tissues were then embedded into paraffin. From each animal five 20μm-thick coronal sections, 100 μm apart from each other were prepared as previously described (Mehra et al., 2005; Mehra et al., 2007). Goat anti-polyvalent IHC Staining Kit (Labvision, Neomaker lab, USA) was used according to manufacturer protocol for the immunohistochemical localization. SYP specific rabbit polyclonal antibody (Santa Cruz Biotechnology, USA) in a 1:200 dilution was used for 48 – 72 hours at 4 °C for the incubation. Sections were then incubated with biotinylated secondary antibody for 24 hours at 4 °C

followed by streptavidin-HRP complex for 2 hours at RT. For proper maintenance of the cytoarchitectural integrity including preventing undesirable background staining, the sections were thoroughly rinsed with wash buffer (0.1M PBS, pH 7.4) between each incubation steps. Localization of the antigen-antibody site was done with the substrate-chromogen reaction using DAB. Immunoreactive sites became brownish under the bright field microscope. Adjacent sections were stained with cresyl violet (CV) to facilitate demarcation of various layers and subfields of the hippocampus. Intermittently some IHC stained sections were also counterstained with CV for the same purpose.

To eliminate non-specific staining, negative controls were processed by incubating the sections with species-specific normal serum, whereas human breast cancer tissue served as the positive controls (data not shown). Sections from all the groups and the immunohistochemical controls were processed simultaneously and repeatedly to rule out any procedural variations.

4.1.4 Image analysis

Semi-quantitative estimation of synaptophysin immunoreactivity (SYP-ir) was carried out on every layer (such as the stratum oriens, stratum pyramidale, and stratum radiatum) in the CA1 and CA2-3 subfields of the dorsal hippocampus in all animals. For the semi-quantitative analysis, integrated optical density (IOD) of SYP-ir was measured using image from five hippocampal sections of each animal, 100 μm apart from each other as previously described (Mehra et al., 2005). These images were viewed under the Nikon Microphot -Fx microscope mounted with a Cool Snap Digital camera (Roper Scientific, USA) and attached to the image analysis system driven by Image Pro-Plus software (v 6.2, Media Cybernetics, USA). The size of the sampling field was 5000 μm^2, where 7-9 non-overlapping digital photomicrographs per section were taken. The quantitative analysis was the same as previously described (Mehra et al., 2005). In brief, photomicrographs were first converted to gray scale with proper background correction, and a standard optical density curve was generated for each image prior to analysis (density of corpus callosum devoid of any pre-or postsynaptic protein was measured as background, and substracted from the image). IOD was measured as cumulative sum of the optical density of immunodense areas. Mathematical values of IOD for comparison between the groups were obtained as arbitrary units (mathematical algoritm) by the analysis software. Data from individual animals of each group were pooled together and the results were expressed as mean IOD ± SD.

4.1.5 Statistics

Statistical analysis was performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego California USA). One-way ANOVA with post-hoc Newman-Keuls Multiple Comparison Test was done to compare mean IOD between groups. The level of significance was set at $p<0.05$.

4.2 Results

4.2.1 Synaptophysin immunoreactivity: Aspect of age

Cell loss in this model of cerebral brain ischemia can be detected both in CA1, and CA3 regions following 10 minutes occlusion, in contrast to the short occlusion times, where only

the CA1 region is affected (Wappler et al., 2010), however, there are surviving cells that gives positive staining to synaptic markers. In the present study, the observed changes in SYP-ir were not limited to just one layer (stratum oriens, stratum pyramidale, or stratum radiatum), but the whole hippocampal region in each case, therefore we discuss our data using the CA1 or the CA3 hippocampal regions.

Young animals showed a significantly lower SYP-ir compared to the old animals after ovariectomy, which resulted in a significant difference between the baseline levels ($p<0.01$ young control vs. old control) (Fig.1., panel A). This might have been caused by the more pronounced change in the circulating estrogen level after ovariectomy in the young than in the old, where the estrogen production of the ovaries is low or there is no estrogen production at all. Due to this difference between the baseline values, age-comparisons were more difficult to make between age groups. Changes are therefore presented as percentages of the age-matched controls (Fig.1., panel B). Old gerbils had more severe synaptic loss in the CA1 area than young gerbils where no significant change following the injury was detected (see 4.2.2. for more details and significant differences among each age group). Estrogen treatment had, however, a positive impact on the synaptic density following ischemia in young animals in the CA3 region (see 4.2.2. for more details and significances between treated and non-treated groups), whereas, the same treatment was less effective in the old gerbils, but still helped imporved SYP-ir to a certain extent (Fig.1., panel B).

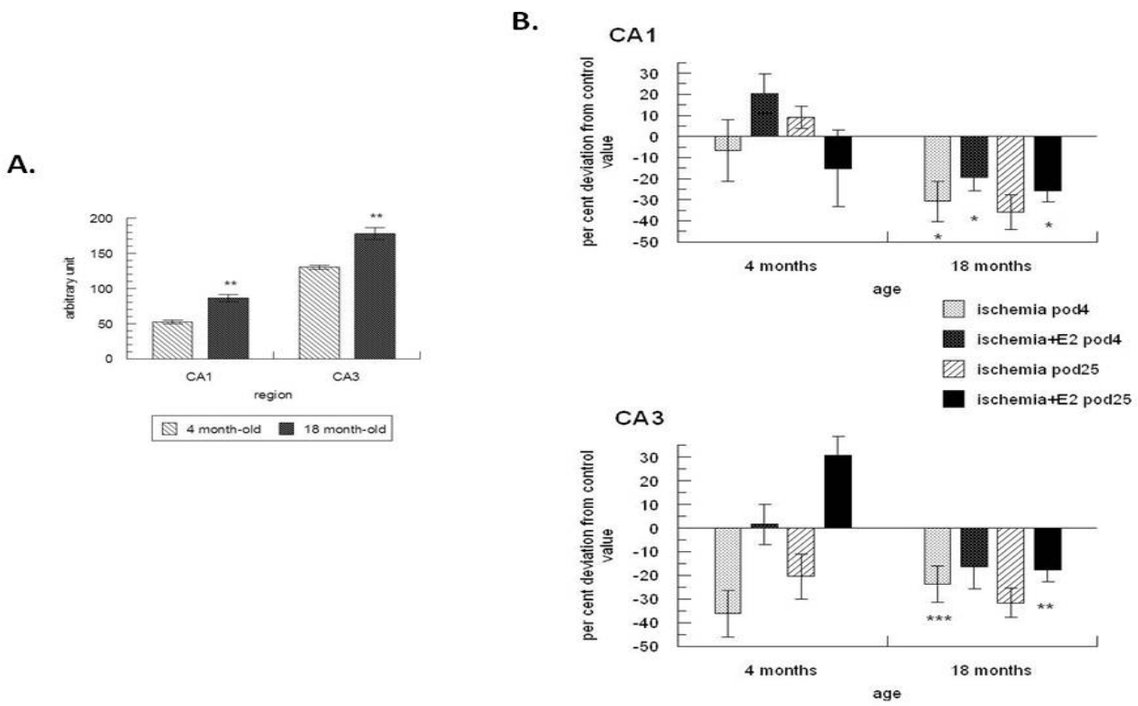

Fig. 1. SYP-ir in the gerbil hippocampus after global cerebral ischemia and estrogen treatment: age comparison. Panel A: control (OVX) groups. Data are expressed as means±SEM. See detailed description in the text. **$p<0.01$ vs. young group same hippocampal region. Panel B: effect of ischemia and ischemia+E2 treatment. Data are expressed as percentages of the age-matched controls±SEM. See detailed description in the text. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. the same treatment group of the 4 months old animals.

Please note that not every significant change is marked on this figure that you can find in the text.

4.2.2 Synaptophysin immunoreactivity: Aspect of time and synaptic regeneration

Cerebral ischemia itself decreased SYP-ir only in the CA2-3 region in the young, but decreased in both CA1 and CA2-3 regions in the old animals. This can be explained by the fact that ovariectomy itself decreases synaptic density in the young animals (Woolley and McEwen, 1992), and not every area is affected the same way. Areas that are more dependent on E2 to preserve their synapses might show a relatively lower decrease after ischemia, as theire baseline synaptic density is very low, and another stress that is not severe enough, can not cause a significant decrease. In contrast, ovariectomy does not make a significant difference in the circulating E2 levels in the old gerbils, however, there is a slight, but progressive loss of synaptic density and an increased vulnerability to ischemia with age (Ojo et al., 2011; Popa-Wagner et al., 2007), the latter causing a significant decrease even compared to a slightly lower control level. In addition, in the young, at the early time point there was an improvement due to E2 pre-treatment in the CA1 region ($p<0.05$ young ischemia POD4 vs. young ischemia+E2 POD4; significance not shown on figure), but at the later time point there was a decrement in synaptic density in the E2-treated group compared to the ischemic group ($p<0.05$ young ischemia POD25 vs. young ischemia+E2 POD25; significance not shown on figure). This can be explained by a higher estrogen-dependency and vulnerability in the CA1 region compared to the CA2-3 region in gerbils. In the old animals ischemia significantly decreased synaptic density in the CA1 region, however, we did not observe significant improvement with the estrogen pre-treatment in POD4. It only was observed at the late time point, which was close to be significant ($p=0.054$ old ischemia POD25 vs. old ischemia+E2 POD25). This is probably because of the decreasing tendency in SYP-ir following ischemia itself by POD25 that made a more prominent difference between the treated and non-treated group, as E2 treatment seemed to preserve the POD4 synaptic density level. We would like to note that there was no signs of recovery in the old animals following ischemia itself as SYP-ir did not icrease by POD25 compared to the POD4 value in the same group.

Moreover, in this study, estrogen pre-treatment increased synaptic density in the hippocampal CA2-3 region in both age groups, however, the increment was more pronounced in the young. The young group showed further increment in synaptic density following ischemia (Fig.2.). In the CA2-3 region at the early and late time point synaptic density increased following estrogen pre-treatment in the young group ($p<0.01$ young ischemia POD4 vs. young ischemia+E2 POD4; and $p<0.001$ young ischemia POD25 vs. young ischemia+E2 POD25; significances not shown on figure), in addition, following ischemia itself SYP-ir also increased in the young in the CA2-3 area ($p<0.05$ young ischemia POD4 vs. young ischemia POD25; significance not shown on figure). However, no significant changes were detected in the old group with the estrogen pre-treatment, and no regenerative changes were observed after ischemia itself either.

4.2.3 Summarizing our results

Even a single dose of E2 treatment can induce long term changes in synaptic density in the injured hippocampus in our gerbil model of cerebral ischemia. At the same time, differences

in age, as well as differences in the investigated brain regions, modulate the degree and the permanence of this E2 effect.

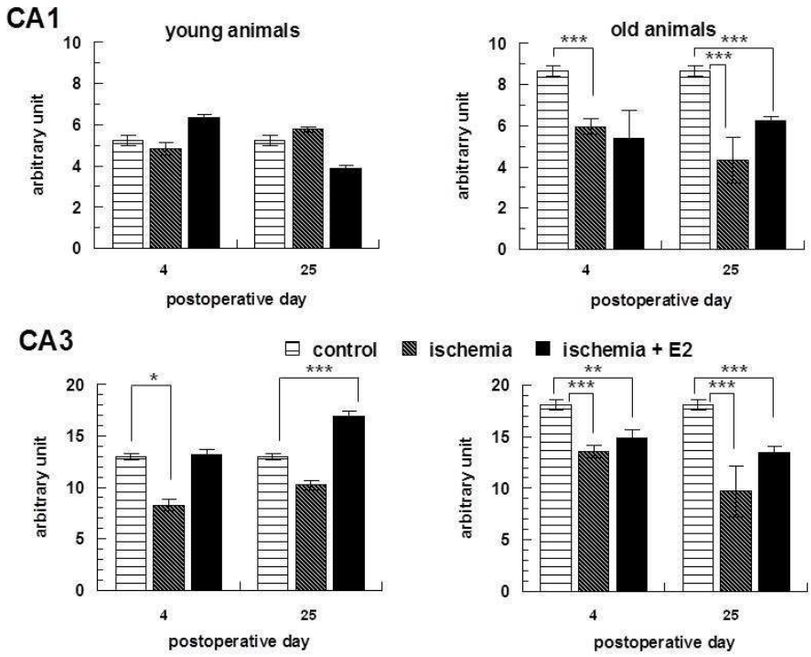

Fig. 2. SYP-ir in the gerbil hippocampus after global cerebral ischemia and estrogen treatment: dynamic changes with time. See detailed description in the text. Data are expressed as means±SEM. *p<0.05, ** p<0.01, ***p<0.001 vs. age-matched control. Please note that only significant changes vs. control groups are shown on the figure, differences between ischemia and ischemia+E2, etc. can be found in the text.

It was an unexpected result for us, that the CA3 region was more vulnerable to ischemia, in terms of synaptic loss, than the CA1 region in the young animals. This result, however, was probably due to the previous ovariectomy that might have had a bigger impact on the CA1 region. This is consistent with our results in the aged animals, where the ischemia decreased synaptic density more in the CA1 region, as expected in this model. It is also possible that aging may predispose to a tendency of diminished synaptogenesis and ability to improve synaptic density, especially in the hippocampal CA3 subfield. Our results emphasize the importance of investigating cerebral regenerative potential in older, female animals as well as at later time points following ischemic injury.

5. Conclusion

Post-ischemic brain regeneration is well documented at the tissue, cellular, and subcellular levels that offer further opportunities for drug development to improve functional outcome. In addition, estrogen, a well known regulator of synaptic density, has a long term impact on regeneration after global cerebral ischemia even as a single, high-dose treatment. Age, however, has influence on its effects, which highlights the importance of using old animals in this field.

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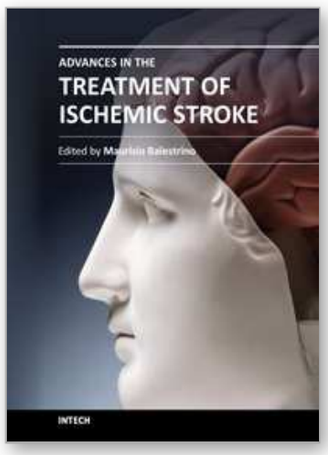

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In recent years research on ischemic stroke has developed powerful therapeutic tools. The novel frontiers of stem cells therapy and of hypothermia have been explored, and novel brain repair mechanisms have been discovered. Limits to intravenous thrombolysis have been advanced and powerful endovascular tools have been put at the clinicians' disposal. Surgical decompression in malignant stroke has significantly improved the prognosis of this often fatal condition. This book includes contributions from scientists active in this innovative research. Stroke physicians, students, nurses and technicians will hopefully use it as a tool of continuing medical education to update their knowledge in this rapidly changing field.

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