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Revisiting the timing hypothesis: Biomarkers that define the therapeutic window of estrogen for stroke

Farida Sohrabji, Amutha Selvamani, and Robyn Balden

Department of Neuroscience and Experimental Therapeutics, Women's Health in Neuroscience Program, Texas A & M Health Science Center, College Station, TX 77843-1114

Abstract

Significantly extended life expectancy coupled with contemporary sedentary lifestyles and poor nutrition have created a global epidemic of cardiovascular disease and stroke. For women, this issue is complicated by the discrepant outcomes of hormone therapy (HT) for stroke incidence and severity as well as the therapeutic complications for stroke associated with advancing age. Here we propose that the impact of estrogen therapy cannot be considered in isolation, but should include age-related changes in endocrine, immune, and nucleic acid mediators that collaborate with estrogen to produce neuroprotective effects commonly seen in younger, healthier demographics. Due to their role as modulators of ischemic cell death, the post-stroke inflammatory response, and neuronal survival and regeneration, this review proposes that Insulin-like Growth Factor (IGF)-1, Vitamin D, and discrete members of the family of non-coding RNA peptides called microRNAs (miRNAs) may be crucial biochemical markers that help determine the neuroprotective “window” of HT. Specifically, IGF-1 confers neuroprotection in concert with, and independently of, estrogen and failure of the insulin/IGF-1 axis is associated with metabolic disturbances that increase the risk for stroke. Vitamin D and miRNAs regulate and complement IGF-1 mediated function and neuroprotective efficacy via modulation of IGF-1 availability and neural stem cell and immune cell proliferation, differentiation and secretions. Together, age-related decline of these factors differentially affects stroke risk, severity, and outcome, and may provide a novel therapeutic adjunct to traditional HT practices.

Stroke is the third largest cause of mortality and the leading non-martial cause of long-term disability in the US. Most recent studies agree on two major trends: stroke incidence increases with age (Roger et al., 2011) and among the elderly, women are more likely to sustain a stroke than men (Petrea et al., 2009; Appelros et al., 2009; Persky et al., 2010), with a surge as early as midlife (Towfighi et al., 2007). The relative stroke protection observed in younger, premenopausal women is usually attributed to ovarian hormones (estrogens and progestins), however, estrogen therapy to older women, paradoxically, increases their risk for stroke.

While the effect of hormone therapy on reducing hip fracture and decreasing the risk for colon cancer are well-established, the crux of the controversy regarding estrogen and hormone therapy has centered on cardiovascular disease (CVD) and stroke due to its impact

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Correspondence to: Farida Sohrabji, Texas A&M Health Science Center Department of Neuroscience and Experimental Therapeutics Medical Education and Research Building 8447 State Highway 47 Bryan TX 77807-3260 Phone: 979-436-0335
sohrabji@medicine.tamhsc.edu.

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on mortality and quality of life. In 1997, a mathematical model of several epidemiological studies concluded that the benefit from CVD provided by hormone therapy far outweighed the potential risks and recommended a broader use of hormone therapy in postmenopausal women (Col et al., 1997). Scarcely a decade later, the Women's Health Initiative (WHI) study yielded just the opposite recommendation, namely that hormone therapy should be given in the smallest dose possible for the shortest amount of time and should not be prescribed for cardiovascular protection (FDA statement posted 8/13/2002). The current dilemma, therefore, is reconciling the overwhelming beneficial effects conferred by estrogen in animal models of stroke with the findings of clinical trials. One of the main hypotheses to emerge from these efforts is the *timing* hypothesis, which proposes that hormone therapy is more benign for stroke when taken by younger women or during the peri-menopausal or early post-menopausal period, but deleterious when taken by women significantly past the menopause (Barrett-Conner, 2007; Choi et al., 2011).

While the timing hypothesis reconciles the disparate findings of hormone therapy, testing this hypothesis is hampered by the lack of an operational definition of timing. Most authors agree that it constitutes a certain period of time during the perimenopause, however, a more specific biochemical signature would be critical for designing testable studies and for eventually tailoring medical practice. Animal studies provide the best avenue for developing markers of the critical period for hormone effectiveness. This review forwards the hypothesis that the effectiveness of estrogen and hormone therapy is modulated by the overall endocrine status of the individual. Here we propose that the gradual loss of effectiveness of estrogen as a neuroprotectant is due to age-dependent loss of other endocrine agents that complement or collaborate with estrogen. Specifically, reduced bioavailability of the peptide hormone IGF-1, or its regulatory modifiers, may define the transition between estrogen as neuroprotectant and estrogen as a neurotoxicant.

To develop this idea, the following paragraphs will review the evidence for estrogen and hormone therapy from the human literature and the experimental studies, followed by the case for IGF-1 as a neuroprotectant and its interaction with estrogen. Finally, we will review other age-related changes in the endocrine environment that impact stroke and IGF-1, focusing specifically on Vitamin D and a novel class of nucleic acid modulators called microRNA.

The impact of timing of hormone therapy on stroke risk

Two types of studies shed light on the issue of timing of hormones and stroke risk: the first compares the risk of stroke due to oral contraceptive (OC) use (which is mainly used by younger women) with hormone therapy use in postmenopausal women and the second consists of subanalyses of postmenopausal women who took HT close to the age of menopause versus those who took HT more than a decade after menopause. With respect to the first category, data on the effect of oral contraceptives for stroke risk in younger women is confounded by the fact that stroke incidence is relatively small in this group. A meta-analysis of 16 studies on stroke risk and OC use concluded that while there was an overall increased risk for stroke among OC users, the absolute risk was considered small since stroke is relatively rare in this population (Gillum et al., 2000). Hypertension, diabetes mellitus, hypercholesterolemia and smoking were much larger contributing factors for stroke in this population as compared to OC use (Balci et al., 2011). Cumulative data on OC and stroke risk is further complicated by the fact that OC formulations have changed over the last 50 years. For example, low dose (<50 micrograms) OC did not affect the odds ratio but high doses of OC increased stroke risk five fold (World Health Organization Study, 1996). Additionally moderate estrogen doses with third generation progestins significantly reduced stroke (Lidegaard 1998).

Hormone use and stroke incidence in postmenopausal women presents a more complicated picture. An early case-control study reported no increased risk for stroke in postmenopausal women who took hormone therapy (Petitti et al., 1998). In a multicenter case-controlled study, increased life time exposure to estrogen was associated with a lower risk of stroke, but interestingly a lower age at menarche increased the odds of stroke (de Lecinana et al., 2007). The WHI study indicated that hormone use increased stroke risk. In this randomized, double blind, placebo-controlled multicenter trial, conjugated equine estrogens (CEE) (Hendrix et al., 2006) and CEE+progestins (Wassertheil-Smoller et al., 2003) increased the risk for stroke. Subgroup analyses indicated that most of this risk was seen in the older age groups. In the CEE trial, increased risk for stroke was statistically significant for the 60-69 yr old group but not in the 50-59 yr old group (Hendrix et al., 2006). In an observational analysis of postmenopausal women in the Nurse's Health Study, estrogen and estrogen +progestin use increased the risk of stroke irrespective of the age of the user or time since menopause (Grodstein et al., 2008). However, the observational arm of the WHI study showed no increase risk for stroke in the CEE or CEE+progestin arm (Prentice et al., 2005; Prentice et al., 2006). A possible factor in the discrepancy between the WHI trial and the WHI observational study was that the initiation of hormones was much earlier in the latter study. It should also be noted that users randomized to the HT arm differ in important physiological traits from women who seek HT independently. For example, an observational trial (SHOW study) reported no difference in stroke risk in HT users, however, HT users were more likely to be normotensive and lean as compared to non-users in this study (Bushnell, 2009), which was not the case in the WHI study, where hypertension incidence was similar in CEE users and non-users (Hendrix et al., 2006). A similar interaction between HT and hypertension was seen in the Danish Nurses study, where the risk for stroke was most prominent among hypertensive women who used hormone therapy (Lokkegaard et al., 2003), while normotensive women who used hormone therapy were no different from controls.

Other evidence for the timing hypothesis also comes from a prospective study of Swedish women, where stroke risk was decreased in women who initiated hormone treatment prior to menopause (Li et al., 2006). In a population-based nested case-control study of 50-69 old women, HT did not significantly elevate ischemic stroke (Arana et al., 2006), further supporting the idea that HT at ages closer to the menopause may be harmless for stroke. Coronary artery calcification, a surrogate marker of cardiac disease, was reduced by estrogen in the youngest cohort of WHI (50-59 years) (Manson et al., 2007), signifying that estrogen's effects can be modulated by the age of the user. Finally, a study of non-HT users found that stroke-related mortality in women 65 and older was higher in women with higher levels of endogenous estrogen (Maggio et al., 2009), implying that elevated levels of hormones in late life, whether exogenous or endogenous, may exert a deleterious effect on stroke.

Animal models of stroke and estrogen treatment

Although experimental stroke studies do not assess risk, they have been instrumental in determining the effect of estrogen on stroke severity. Similar to human stroke, female sex appears to be protective, such that females have a smaller infarct and better cerebral blood flow than age-matched males both in normoglycemic (Alkayed et al., 1998) and diabetic (Toung et al., 2000) animals and this advantage is lost when females are ovariectomized. Estrogen replacement to normoglycemic females is neuroprotective, but estrogen replacement to diabetic mice (db/db) did not reduce infarct size or regulate apoptotic genes (Zhang et al., 2004).

Stroke injury to females in proestrus (high estrogen levels) result in smaller infarcts than those in metestrus (low estrogen state) and the extent of ischemic damage was inversely related to circulating levels of estrogen (Liao et al., 2011). Bilateral ovariectomy worsens infarct volume and longer periods of estrogen deprivation (1 week versus 4 week of ovariectomy) further increase the size of the infarct (Fukuda et al., 2000). Direct neuroprotective effects of estrogen in reducing infarct volume and mortality have been reported for 17 β estradiol and its inactive stereoisomer 17- α estradiol (Simpkins et al., 1997) and CEE (McCullough et al., 2001). Exogenous estrogen replacement is neuroprotective when given prior (Dubal et al., 1998) or subsequent to the injury (Liu et al., 2007; Yang et al., 2003), and is also effective in males (Toung et al., 1998).

However, estrogen replacement is not universally protective. Estrogens neuroprotective effects are more consistently seen in cortical areas rather than sub cortical regions, although in a less severe stroke (30 min ischemia versus 60 min ischemia), estrogen may also protect subcortical damage (Fan et al., 2003). Estrogen replacement to the Wistar-Kyoto rat strain reportedly increases infarct volume (Carswell et al., 2004). In view of the observation that hypertension in women may modulate the effect of estrogen on stroke risk, it is worth noting that estrogen replacement had no protective effect on infarct size in the stroke prone spontaneously hypertensive rat (Carswell et al., 2004). In an instance of severe ischemic injury, where cerebral vessels (single middle cerebral artery [MCA] and both common carotids) were occluded for 3h, there were no gender differences in infarct size and no reduction of the infarct due to intravenous or subcutaneous 17 β -estradiol (Vergouwewen et al., 2000). Based on their studies where estrogen fails to protect against stroke in the ovariectomized spontaneously hypertensive stroke prone rat (Carswell et al., 2004), and actually increases damage in the Wistar-Kyoto rats, Lister Hooded and Sprague–Dawley rats (Bingham et al., 2005; Gordon et al., 2005), Macrae and Carswell (2006) have suggested that the neuroprotective effect of estrogens may be less effective in permanent ischemic models.

Since stroke occurs primarily in an older population, the role of estrogens in older animals is vital to predicting its role as a neuroprotectant. Relatively few studies have assessed older females in experimental stroke models. Stroke damage is worse in older females as compared to younger females (Takaba et al., 2004; Liu et al., 2009, Selvamani and Sohrabji, 2010a), however studies differ in their conclusions regarding estrogen treatment to older females. Estrogen replacement is neuroprotective in both young and middle-aged females in the MCAo suture stroke model (Alkayed et al., 2000; Dubal and Wise 2001), although estrogens failed to attenuate hippocampal cell death in a bilateral carotid artery occlusion model in middle-aged gerbils (De Butte-Smith et al., 2007). In middle-aged female rats, characterized as reproductively senescent by daily vaginal smears and with virtually undetectable levels of estrogen (constant diestrus), estrogen treatment increased infarct volume and worsened sensory motor performance in an endothelin-1 vasoconstriction model, although estrogen treatment to multiparous young females was neuroprotective in this model (Selvamani and Sohrabji, 2010a; 2010b). Hence, estrogen treatment to older females is less likely to be neuroprotective, and this may be linked to the severity of the stroke model or to differences in the definition of middle age. Middle-aged animals defined as reproductively senescent have experienced a period of hypoestrogenicity and this may make their response to subsequent estrogen treatment less favorable. In this reproductively senescent group, estrogen treatment failed to reduce infarct volume when subject to the MCAo suture stroke model (Selvamani and Sohrabji, unpublished observations), suggesting that a period of hypoestrogenicity may be more critical than the type of stroke model. This is further supported by a study by Wise and colleagues where ovariectomized females that were replaced with estrogen either immediately or 10 weeks later. Immediate estrogen replacement reduced infarct volume and attenuated the inflammatory response, while

estrogen replacement after a prolonged period of hypoestrogenicity was ineffective (Suzuki et al., 2007).

Collectively, the timing hypothesis receives some support from experimental studies, and the age-dependent effect of estrogen in the vasoconstrictive model provides a useful model to explore the process or event that determines whether estrogen will exert a positive or negative effect. In the endothelin-1 vasoconstrictive stroke model, we reported that estrogen was neuroprotective to adult females but neurotoxic to acyclic middle aged females (Selvamani and Sohrabji, 2010b). This discrepancy suggests that compensatory changes that accompany reproductive senescence or menopause create an environment where exogenous estrogen is no longer neuroprotective. Besides the decline of ovarian hormones, several other endocrine systems display age-related changes including testosterone, dehydroepiandrosterone, and the growth hormone (GH)/IGF-1 axis (Lamberts et al. 1997). Each of these hormones and additional others such as glucocorticoids and Vitamin D exhibit a wide variety of neuroprotective and immune-modulating capabilities (Heffner 2011). Age-related changes in endocrine regulation of neuronal health and immune-mediated inflammatory processes may act synergistically to alter the effects of hormone therapy. Estrogen collaborates with a wide variety of tissue specific growth factors to promote cell survival, proliferation and angiogenesis and in the brain, we propose that estrogens neuroprotective actions are dependent on the availability of insulin-like growth factor (IGF)-1, and an age-related decline of IGF-1 thus alters the outcome of estrogen therapy in older animals and women.

The case for IGF-1

The Growth Hormone (GH)/Insulin-like Growth factor-1 axis exhibits remarkable age-related changes, and this phenomenon, known as “*somatopause*”, causes reductions in lean body mass, bone mass and immune function. Aging reduces the levels of circulating IGF-1 and estrogen replacement further reduces circulating IGF-1 levels in young and middle-aged females (Bottner and Wuttke 2006; Selvamani and Sohrabji, 2010b).

Although the liver is the single largest source, diverse organs including the brain synthesize IGF-1 (Baskin et al, 1988) and IGF-1 has specific growth promoting actions on several cell types. In breast cancer cells, estrogen/IGF-1 cross talk has been shown to influence cell proliferation (Dupont and Le Roith 2001; Martin and Stoica, 2002). Such synergy is also seen in the brain, where estrogen and IGF-1 receptors are co-localized to the same cell (Quesada et al., 2007), promote the survival (Traub et al., 2009; Quesada et al., 2008) and differentiation of the same groups of neurons and stimulate adult neurogenesis (reviewed in Mendez et al., 2005). IGF-1 is produced by both neurons and glia, but has distinct actions on each cell type, producing proliferation in astrocytes (Torres-Aleman et al., 1998), activating PI-3K and Akt signaling in microglia (Strle et al., 2002), while stimulating pro-survival pathways in neurons (Dudek et al., 1997). Exogenous IGF-1 reduces ischemic injury in many species (Gluckman et al., 1992; Lee and Bondy, 1993; Johnston et al., 1996; Guan et al., 2001), stimulates stroke-induced neurogenesis (Yan et al., 2006) and promotes neuronal survival, neuronal myelination and angiogenesis (Smith, 2005; Wang et al., 2000). Intravenous (Rizk et al., 2007), intracerebrovascular, intranasal and adeno-assisted virus delivery (Lin et al., 2009) of IGF-1, all improve stroke outcomes, indicating versatile therapeutic options for this peptide. Thus, the almost-universal decline in circulating IGF-1 levels with aging (Waters et al., 2003; Denti et al., 2004) which is thought to increase longevity (Brown-Borg et al., 2007; Bartke et al., 2003), may be deleterious for the injured brain.

In observational studies, lower levels of circulating IGF-1 were predictive of a poorer outcome for stroke patients (Bondanelli et al., 2006; Johnsen et al., 2005, De Smedt et al.,

2011; Aberg et al., 2011, but see Endres et al., 2007) and increased mortality (Denti et al., 2004; van Rijn et al., 2006), supporting the neuroprotective effects reported for this peptide in experimental studies. Furthermore, low levels of the IGF-1 binding protein (IGFBP)-1 are associated with coronary events (Kaplan et al., 2007) and ACE-inhibitor therapy to older adults with CVD risk is associated with increased IGF-1 and IGFBP-3 (Giovannini et al., 2010).

A critical substrate for the neuroprotective effects of IGF-1 as well as other endocrine hormones is the immune system. The post-stroke inflammatory response plays a critical role in the pathophysiology of cerebral ischemia (Kreutzberg 1996; Hill et al. 1999; Vila et al. 2000; Chapman et al. 2009), and is affected by IGF-1 as well as estrogen (Czlonkowska et al. 2006; Strom et al. 2011). Cerebral ischemia results in a characteristic post-stroke inflammatory response, involving activation of astrocytes and immune cells, increased vascular and blood brain barrier (BBB) permeability, influx of leukocytes, and cytokine production (del Zoppo et al. 2000; Becker 2001; Rothenburg et al. 2010). While the post-stroke inflammatory response is necessary and beneficial to neuronal survival (Kreutzberg 1996; Denes et al., 2010a), infiltration of certain T cell subsets may promote brain injury, while others are thought to provide neuroprotective benefits (Kreutzberg 1996; Wang et al., 2007; Denes et al. 2010b; Yilmaz and Granger 2010). In addition to its role as a neuroprotectant, IGF-1 is also known to shape the immune environment of the ischemic brain. IGF-1 exerts proliferative effects on T cells (Clark et al. 1993) and adult neural stem cells (Gonzalez-Perez et al. 2010a), and IGF-1 signaling influences the anti-proliferative action of IFN- γ on polarized Th1 cells (Conti et al. 2007). Adult neural progenitor cells (NPCs) are an important component of post-stroke tissue repair and regeneration, and local cytokine, chemokine, and growth factor expression regulates the survival, neuroprotective efficacy, and fate of these cells (Gonzalez-Perez et al. 2010b; Gonzalez-Perez et al. 2010a). IGF-1 increases neurogenesis and is one of the most studied immunological effectors of neural stem cells, while pro-inflammatory cytokines IFN- γ and TNF- α reduce neural progenitor cell (NPC) proliferation and survival (Gonzalez-Perez et al. 2010a). Communication between immune cells and NPCs at the site of ischemic injury promotes a microenvironment conducive to tissue repair and regeneration including cytokine expression, trophic support, and neuronal repopulation. Additionally, NPCs can differentiate into classically neuroprotective CNS support cells including astrocytes and oligodendrocytes (Gonzalez-Perez et al. 2010b).

IGF-1/estrogen interactions

IGF-1 and estrogen act synergistically in experimental models of neurodegenerative diseases (Garcia-Segura et al., 2000; 2006). Both prevent excitotoxin-induced cell death in hilar neurons (Azcoitia et al., 1999), and the actions of one receptor are inhibited by antagonists to the other's receptor. Thus, 6-hydroxydopamine (OHDA) lesions targeting the nigrostriatal dopaminergic pathway are attenuated by either estrogen or IGF-1, while the IGF-1 inhibitor JB-1 can inhibit the protective actions of both estrogen and IGF-1 (Quesada and Micevych, 2004). In fact, IGF-1 can activate estrogen receptors in the absence of estradiol (Ma et al., 1994). Both *in vivo* (El-Bakri et al., 2004) and *in vitro* studies show that estrogen can regulate the IGF-1 receptor (Cardona-Gomez et al., 2002). *In vivo*, estrogen transiently activates the IGF-1 receptor through tyrosine phosphorylation, leading to an interaction of estrogen receptor (ER)-alpha with IGFR-1 and a greater interaction with components of the PI3K pathway (Mendez et al., 2003).

If IGF-1 is critical for modulating estrogen's effects, then age-related decline in IGF-1 levels would reduce estrogen's neuroprotective capacity. We tested this hypothesis using adult and middle aged female rats, with the endothelin-1 vasoconstrictive stroke model, where we reported that estrogen was neuroprotective for stroke in young ovariectomized females, but

neurotoxic to middle aged females (Selvamani and Sohrabji 2010a). Consistent with our hypothesis, we found that IGF-1 was reduced in plasma from middle-aged animals as compared to young females (Selvamani and Sohrabji 2010b). We then injected middle aged females with recombinant IGF-1 (intracerebroventricular (ICV) delivery) 4h post stroke and found that estrogens neurotoxic effects on stroke were reversed, resulting in greater tissue protection in estrogen treated middle-aged animals as compared to controls treated with or without IGF-1. This data suggests that reduced IGF-1 bioavailability may impair estrogen's actions in older animals. Furthermore, we found that injections of the IGFR antagonist, JB-1, abrogated the neuroprotective effect of estrogen in young females, such that estrogen treated young females given JB-1 now had a larger infarct as compared to estrogen-deprived controls, indicating that estrogen-IGF-1 interactions are critical for estrogen-mediated neuroprotection at both ages. Estrogen/IGF-1 heterodimers can be detected in the ischemic brain and IGF-1/estrogen treatment suppressed ischemia-induced ERK activation, while estrogen alone or IGF-1 alone did not (Selvamani and Sohrabji, 2010b). These studies support our hypothesis that reduced IGF-1 availability may signal the end of the neuroprotective window of estrogens beneficial effects.

Regulatory influences on IGF-1

Reduction of IGF-1 in aging is almost universal, occurring in worms to humans, and understanding the regulatory controls on this peptide would help maintain the neurotropic potential of estrogen and provide new therapeutic targets for stroke. Growth hormone (GH) is a critical mediator of IGF-1 levels and could be used to transiently elevate IGF-1 availability after stroke. However, GH treatment may also cause edema, hypertension, hyper-insulinemia and impaired glucose tolerance. In this context, other members of the steroid receptors superfamily of ligand-activated transcription factors, such as Vitamin D may be particularly important.

Vitamin D

Similar to estrogen, Vitamin D upregulates neurotrophic factors such as nerve growth factor (NGF) (Neveu et al. 1994), insulin-like growth factor (IGF-1) (Bogazzi et al. 2011), and the glial-derived growth factor GDNF (Naveilhan et al. 1996) in the brain. Additionally, circulating levels of IGF-1 are positively correlated with Vitamin D levels (Bogazzi et al. 2011), and negatively correlated with ischemic stroke outcome in experimental and clinical studies (Gluckman et al. 1992; Schwab et al. 1997; Denti et al. 2004; Zhu et al. 2009). Furthermore, Vitamin D deficiency (VDD) is clinically associated with dysfunction or failure of the insulin/IGF-1 axis in cardiovascular disease states such as diabetes and metabolic syndrome that increase the risk and severity of stroke (Botella-Carretero et al. 2007; Towfighi and Ovbiagele 2008; Alvarez and Ashraf 2010; Bogazzi et al. 2011).

If estrogen's actions on stroke are influenced by other cardiovascular risk factors such as hypertension (discussed above), then decreased levels of Vitamin D in postmenopausal women may also contribute to estrogen's diminishing neuroprotection and increased stroke risk. Low Vitamin D levels have been implicated in numerous chronic inflammatory conditions including cardiovascular disease, cancer, autoimmune, and infectious diseases (Cantorna and Mahon 2004; Bouillon et al. 2008; Lee et al. 2008; Bikle 2009; Bartley 2010; Bouvard et al. 2011), and are associated with a greater risk for stroke. Critical risk factors for cerebral infarction such as age and hypertension are also associated with low serum 25-OHD, the circulating form of Vitamin D (Lappe et al. 2006; Scragg et al. 2007; Pilz and Tomaschitz 2010), and low serum 25-OHD was independently predictive of future strokes in a number of clinical studies (Marniemi et al. 2005; Pilz et al. 2008). IGF-1 promotes the hydroxylation of 25-OHD₃ into the active 1,25(OH)₂D₃ hormone by inducing renal 1- α -hydroxylase, the enzyme responsible for vitamin D activation (Gomez 2006). Conversely,

Vitamin D elevates IGF-binding proteins (IGFBPs) (Gomez 2006; Hypponen et al. 2008), which are thought to protect IGF-1 from proteolytic cleavage, and interactions between VDR and the IGF binding proteins (IGFBPs) have been reported (Cui et al. 2011). This evidence is consistent with the overall hypothesis of this review, which proposes that age-related decline of IGF-1 and IGF-1 regulatory elements may underlie the gradual loss of estrogen's effectiveness in postmenopausal women, a group especially at risk for stroke.

Estrogen, Vitamin D and IGF-1 actions may converge on the immune system. Estrogen is a known immunomodulator that suppresses inflammatory processes and adaptive immunity (Carlsten 2005), while IGF-1 exerts proliferative effects on immune cells and neural stem cells (Clark et al. 1993; Gonzalez-Perez et al. 2010a). Vitamin D is known to act directly and indirectly as a potent immunomodulator (Manolagas et al. 1985; Cantorna et al. 2000; Cantorna et al. 2004; Schleithoff et al. 2006; Bouillon et al. 2008), capable of influencing T cell and antigen presenting cell activation, differentiation and phenotype, function, and secretions (Bikle 2009; Kamen and Tangpricha 2010). Activated Vitamin D generally enhances innate and anti-inflammatory immune function and cytokine expression, while adaptive and pro-inflammatory processes and cytokines are largely suppressed (Barrat et al. 2002; Gregori et al. 2002; Daniel et al. 2008; Bikle 2009; Maruotti and Cantatore 2010) and in so doing, Vitamin D also prevents excessive immune responses, which are deleterious in stroke. Additionally, activated Vitamin D, or 1,25(OH)₂D₃, upregulates anti-microbial peptides in response to infection (Kamen and Tangpricha, 2010), and by appropriately stimulating monocyte/macrophage differentiation and maturation (Hewison, 2010). VDD is thus particularly deleterious in the context of cerebral ischemia because post-stroke infections are a primary cause of mortality in acute stroke patients. Furthermore, cognitive abilities decline with age, particularly memory and spatial learning, and have been shown to be highly dependent on immune modulation (Ron-Harel and Schwartz, 2009). Immune-dependent maintenance of brain plasticity is mediated by signals that improve glial cell sensitivity and response to homeostatic deviations and the upregulation of growth factors such as BDNF and IGF-1 (Ron-Harel and Schwartz, 2009). Our recent work shows that diet induced Vitamin D deficiency (VDD) not only increases infarct volume and exacerbates loss of sensory motor function in female rats, but furthermore shows that VDD-induced stroke severity is accompanied by decreased IGF-1 in the ischemic brain, and elevated levels of the cytokines IL-6 and TGF-beta (Balden et al., 2012). By promoting pro-inflammatory cytokine profiles, VDD facilitates development of neurotoxic excessive immune responses.

Other labs have reported a neurotoxic role for Vitamin D deficiency (Cekic et al., 2009a) and have shown neuroprotection in animal models of stroke by pretreating with Vitamin D (Wang et al., 2000, Yasuhara et al., 2008, Ekici et al., 2009) or acutely administering Vitamin D in combination with other neuroprotective hormones such as progesterone post-ischemia (Cutler et al., 2007, Cekic et al., 2009a, Cekic et al., 2009b). *In vitro*, Vitamin D₃ protects rat cortical neurons (Taniura et al., 2006) and retinal ganglion cells (Suemori et al., 2006) against glutamate-induced neurotoxicity, and Vitamin D further activates CD4⁺ T cells, which have been shown to facilitate glutamate uptake by astrocytes in ischemic tissue (Garg et al., 2008). Furthermore, Vitamin D enhances endogenous anti-oxidant pathways such as γ -glutamyl transpeptidase (Garcion et al., 1996) and reduces inducible nitric oxide synthase (iNOS) (Garcion et al., 1997), suggesting a mechanism for Vitamin D-induced neuroprotection.

Estrogen loss and Vitamin D deficiency intersect to alter the risk for stroke. In patients with concomitant metabolic syndrome and reduced bone mineral density or osteoporosis, markers of inflammatory disease and stroke severity such as C-Reactive Protein (CRP) and high sensitive CRP, were significantly higher in postmenopausal women as compared with premenopausal women (Jeon et al. 2011), but not with age-matched men (Lee et al. 2011).

Clinical trials have shown that Vitamin D reduces inflammatory cytokines and levels of CRP (Zittermann, 2003), while Vitamin D deficiency is associated with metabolic syndrome in morbid obesity (Botella-Carretero et al. 2007). Results from the National Health and Nutrition Examination Surveys (NHANES) 1988-1994 versus 1999-2004 revealed that a tripled stroke prevalence in women age 35-64 was accompanied by increased prevalence of a number of metabolic syndrome components, while stroke prevalence was stable in men (Towfighi et al. 2010). Vitamin D deficiency is associated with reduced IGF-1 levels and insulin resistance, and thereby indirectly exacerbates conditions such as diabetes and metabolic syndrome that are characterized by insulin/IGF-1 axis dysfunction or failure (Michos and Melamed, 2008, McColl et al., 2009). Due to the known neuroprotective and regenerative properties of IGF-1 in cerebral ischemia, it is not surprising that diabetics experience increased stroke severity and mortality. Furthermore the window of viability for penumbral tissue, the area immediately surrounding the infarct, is reduced in experimental animal models of hyperglycemia (Baird et al., 2002), and VDD is highly correlated with the incidence of hyperglycemia, insulin resistance, and diabetes.

Together, these findings suggest that risk factors for CVD and stroke that are related to Vitamin D deficiency as well as the individual's hormonal status differentially influence stroke risk and outcome. It further suggests that IGF-1 regulation lies at the intersection of this health risk, although more research is needed in this area.

Nucleic acid regulation of IGF-1

Although steroid hormone regulation of IGF-1 is well recognized, peptide regulation by non-coding RNA is only recently documented and less well-studied. MicroRNAs, a subset of small non-coding RNA (*miRNAs*) are 18-25 nucleotide-length, non-coding RNA molecules, that are important regulators of mRNA transcript stability (Denli and Hannon, 2003) and gene translation (Ambros, 2001), and their wide effects on the proteome have earned the term nucleic acid hormones. Stroke alters a large number of miRNA in both human (Liu et al., 2010) and experimental (Dharap et al., 2009; Jeyaseelan et al., 2008) models, and specific microRNA may serve as biomarkers for presence or severity of stroke. Plasma levels of miR210 discriminate between ischemic and control patients and correlates well with stroke outcomes (Zeng et al., 2011). MiR124, a brain specific microRNA which declines following stroke in progenitor cells of the SVZ cells, is also reported to be a biomarker for cerebral infarction in an MCA occlusion model (Weng et al., 2011).

Due to its role in both aging and stroke, microRNA control of the IGF-1 signaling system has received considerable attention. Both miR71 and miR239 which predict longevity in *C. elegans* target insulin/IGF-1 signaling elements (Pincus et al., 2011). MiR7 has been shown to regulate IGFR-1 in tongue squamous carcinoma cells. (Jiang et al., 2010) and miR320 regulates IGF-1 in myocardial microvascular endothelial cells (Wang 2009). Perhaps the best-studied IGF-1-associated microRNA is miR1. MiR-1 has been shown to decline with age in *C. elegans* and its disappearance has been correlated with muscle aging (Ibanez-Ventoso et al., 2009). However, only 7% of *C. elegans* survive to day 15, which suggests alternatively, that declining miR-1 may be a compensatory process that promotes survival. MiR1/206 is also known to be a tumor suppressor microRNA and is consistently down regulated in prostate cancer (Hudson et al., 2012). Consistent with its role as a translational repressor, several MiR1 actions appear to antagonize those of the IGF-1 signaling system, such as proliferation and muscle growth. For example, miR1 has been shown to reduce the pool of proliferating cardiomyocytes (Zhao et al., 2005), inhibit smooth muscle cell proliferation (Chen et al., 2011), decreased skeletal muscle hypertrophy (McCarthy and Esser, 2007). Conversely, suppression of MiR1 results in cardiac hypermyotrophy (Li et al., 2010). MiR1 is extensively associated with cardiac tissue. MiR1 is the most abundant microRNA in the heart and serum levels of MiR1 are rapidly increased after acute

myocardial infarction and is correlated with infarct volume. In patients with myocardial infarction, miR1 is elevated in blood and correlates with creatine kinase (Cheng et al., 2010). Using a bioinformatics driven approach, Elia and colleagues (2009) showed that miR1 targets IGF-1 and the IGF-1 receptor, and that miR1 and IGF-1 reciprocally regulate the other. Furthermore, miR1 is strongly negatively correlated with left ventricular mass in acromegaly patients, where IGF-1 is chronically elevated.

Brain expression of miR1 is less well studied, although miR1 has been shown to attenuate neurite outgrowth in DRG neurons in vitro (Bastian et al., 2011). In our studies comparing adult and middle aged females, miR1 was significantly increased in the cortex of middle-aged females (Selvamani et al., 2012), which is consistent with reduced IGF-1 levels seen in this group. Similarly, Let7f, another microRNA with a consensus binding-site in the IGF-1 gene, is also elevated in middle-aged females (Selvamani et al., 2012). We hypothesized that antagonists to IGF-1 associated microRNA could be exploited to improve neurological outcomes in the ischemic brain. Our studies show that anti-Let7f preserved both cortical and striatal tissue and improved performance on sensory motor tasks, when injected ICV (intracerebroventricular) 4h after onset of stroke. Antagomirs to miR1 were also beneficial but had a less dramatic effect than anti-Let7f, resulting in protection of cortical, but not striatal, tissue. Anti-Let7f also increased IGF-1 synthesis in ischemia-activated microglia, which has been shown to promote neuroprotection (Lalancette-Hebert et al. 2009). In the case of Let7f, antagomirs to this miRNA were effective in neuroprotection in females but not males and were also ineffective in ovariectomized females (Selvamani et al., 2012), suggesting that the gonadal hormone environment impacts or amplifies regulation of neuroprotective gene families. Although the underlying mechanism is unclear, it is worth noting that both estrogens and Let7f (www.targetscan.org) target genes in common such as BDNF (Jezierski and Sohrabji, 2000), synaptic proteins (Varju et al., 2009) and synthesizing enzymes for inflammatory mediators such as PGE2 (Frasor et al., 2008).

Conclusion

This review highlights the importance of accounting for multiple endocrine, immune, nutritional, and lifestyle risk factors while evaluating the association between stroke and HT. Estrogen, Vitamin D, IGF-1 and other novel regulatory molecules clearly interact and can result in markedly different biological climates, and the loss of these endocrine agents may serve as a signal for altered effects of hormone therapy with the postmenopausal demographic, especially in the context of stroke.

Regulation of miRNA expression via ligand occupancy of nuclear hormone receptors is a possible key mechanism by which estrogen, IGF-1, and Vitamin D may interact. Activation vs. repression of miRNA expression has been hypothesized to link nutrient availability to developmental programs (Rougvie, 2009), thus presence or absence of the ligand for a particular nuclear hormone receptor may mediate opposing downstream effects depending on steroid hormone availability (Bethke et al., 2009). The consequences of such programming are obvious in the context of aging and declining hormone availability as discussed in this review. Efficacy of hormone replacement should therefore not be evaluated outside of the comprehensive endocrine status of individual patients.

The individual's hormonal status differentially influences stroke risk and outcome, and research suggests that IGF-1 regulation lies at the intersection of this health risk. Vitamin D deficiency and insulin/IGF-1 resistance is characteristic of metabolic disruption and diabetes mellitus, common risk factors for stroke, and the let-7 family of miRNAs has recently been shown to control glucose homeostasis and insulin sensitivity. Mice over-expressing let-7 had impaired glucose tolerance, impaired insulin secretion, and reduced fat mass and body weight (Frost and Olson, 2011). Knockdown of let-7 miRNAs in mice with diet-induced

obesity prevented or restored glucose tolerance in part by improving insulin sensitivity in liver and muscle (Frost and Olson, 2011). Thus, in conditions of reduced Vitamin D and IGF-1 availability, suppression of let-7 miRNA family expression improves metabolic function, while suppression of let-7 miRNA (or other IGF-1 associated miRNA) and consequent elevation of IGF-1, may likely improve the deleterious effects of estradiol deficiency.

Considering the multi-phasic nature of the post-stroke immune response and regenerative processes, it becomes highly likely that miRNA-hormone coupled molecular switches play a critical role in the outcome of cerebral ischemia, and that the presence of pro-inflammatory cytokines can interrupt or dysregulate these molecular switches, resulting in attenuated neuroprotective functionality of estrogen.

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Highlights

- Aging affects the effectiveness of hormone therapy on stroke outcomes in women
- Experimentally, estrogen's effects vary with the animal's age, model of ischemia and comorbid conditions
- IGF-1 availability may predict whether the actions of estrogens are neuroprotective or neurotoxic
- IGF-1 is affected by age as well as the endocrine and nutritional status of the organism
- Convergence of IGF-1, Vitamin D and nucleic acid regulators (microRNA) in stroke outcomes and aging