

## Estrogens and progesterone as neuroprotectants: what animal models teach us

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## 1. ABSTRACT

Estradiol and progesterone are two steroid hormones that target a variety of organ systems, including the heart, the bone and the brain. With respect to the latter, a large volume of basic science studies support the neuroprotective role of estradiol and/or progesterone. In fact, the results of such studies prompted the assessment of these hormones as protective agents against such disorders as Alzheimer's disease, stroke and traumatic brain injury. Interestingly, results from the Women's Health Initiative (WHI) yielded results that appeared to be inconsistent with the data derived from *in vitro* and *in vivo* models. However, we argue that the results from the basic science studies were not inconsistent with the clinical trials, but rather, are consistent with, and may even have predicted, the results from the WHI. To illustrate this point, we review here certain *in vivo* paradigms that have been used to assess the protective effects of estrogens and progesterone, and describe how the results from these animal models point to the importance of the type of hormone, the age of the subjects and the method of hormone administration, in determining whether or not hormones are neuroprotective.

## 2. INTRODUCTION

The U.S. Census Bureau estimates that by 2010, the population of women between 45 and 64 years old will reach approximately 42 million, a marked increase from the value reported for 2000 (approx. 32 million) (U.S. Census Bureau. Projected population of the United States, by Age and Sex: 200 to 2050. [www.census.gov/ipc/www/usinterimproj/](http://www.census.gov/ipc/www/usinterimproj/) Internet release date: March 18, 2004). This increasing number of women will consequently need to make decisions about the use of hormone therapy to treat not only menopausal symptoms, but potentially, to maintain a healthy brain. And though numerous basic science studies, epidemiological studies and some clinical trials have supported the potential benefit of hormone therapy in reducing the incidence of age-associated brain dysfunction (including reducing the risk for Alzheimer's disease), recent results from the Women's Health Initiative (WHI) have suggested the contrary and left the field unsettled as to the future of hormone therapy. For example, caveats of the WHI (particularly the WHI memory study, WHIMS) include the possibility that both the age of the subjects and the duration of post-menopausal

hormone deprivation diminish the protective brain response to steroid hormones. Additionally, the type of hormone (for example, progesterone versus the synthetic, medroxyprogesterone acetate) and its method of administration may have also influenced the outcomes.

While it is clear that a better understanding of the neurobiology of gonadal steroid hormones and their receptors is needed, it is equally important that we interpret the basic science studies appropriately, and understand their limitations if we are to apply the results from these studies towards the design of the next large-scale clinical trial or in fact, implement safer and more effective ways of treating the menopause and the post-menopausal period. To this end, we review here some of the common animal models that have been used to assess the neuroprotective efficacy of estrogens and progestins, and describe some of the limitations that must be acknowledged in interpreting their results.

### 3. MODELS USED TO ASSESS THE NEUROPROTECTIVE EFFECTS OF ESTROGENS AND PROGESTINS

#### 3.1. The ovariectomized rodent

*In vivo* studies that have tested the effects of estrogens and/or progestins on various aspects of neurobiology have often employed the ovariectomized rodent (using either rats or mice) as an experimental model. Indeed, numerous studies have demonstrated that ovariectomy can result in impairment of cognitive performance (1-3), influence structural plasticity of neurons (4), impair cholinergic function (2, 5), and reduce neurotrophin expression (3, 6-8). All these changes have been linked to such neurodegenerative diseases such as Alzheimer's disease, and as such, the ovariectomized rodent has been used to assess if steroid hormones such as estradiol and progesterone may prevent or attenuate some of these deficits. In fact, both estradiol and progesterone have been shown to be effective neuroprotectants in a variety of animal models in which ovariectomy was used to eliminate ovarian steroid hormone production. Examples are provided below:

#### 3.2. The stroke model

The neuroprotective effects of estrogens have been demonstrated in a variety of models of stroke, as induced by causing acute cerebral ischemia. These include transient and permanent middle cerebral artery occlusion models (9-11), global forebrain ischemia models (12, 13), photothrombotic focal ischemia models (14), and glutamate-induced focal cerebral ischemia models (15). The neuroprotective effects of estrogens have also been demonstrated against subarachnoid hemorrhage, a highly prevalent form of stroke in females (16). These protective effects have been described in multiple species, including rats, mice and gerbils (17, 18). Collectively, these results support the argument that estrogens could be valuable candidates for brain protection in females.

It is important to recognize that ovariectomy causes a dramatic reduction in not only circulating

estradiol, but also in circulating progesterone. As such, the structural and functional impairments that are reported to occur following ovariectomy may result from the loss in not only circulating estrogens, but of progesterone as well. Accordingly, it should come as no surprise, that progesterone is also an effective neuroprotectant in animal models of stroke. For example, Jiang *et al.* illustrated that the administration of progesterone before middle cerebral artery occlusion (MCAO) resulted in a marked reduction in cerebral infarction and reduced impairments that resulted from the occlusion (19). Interestingly, progesterone was also found to be protective even when administered shortly after the ischemic event (20, 21), and resulted in improvements in various functional measures, including the rotarod test, and adhesive-backed somatosensory and neurological scores (22). Further, progesterone also protected against cell death following an acute episode of global ischemia (23), and may be mediated by progesterone's ability to reduce lipid peroxidation, the generation of isoprostanes (24) and the expression of pro-inflammatory genes (25).

#### 3.3. Traumatic brain injury

Another *in vivo* paradigm that has taken advantage of the ovariectomized model to assess the effectiveness of steroid hormones as a neuroprotectant is the traumatic brain injury (TBI) model. In particular, studies assessing the effects of progesterone have found that it can significantly reduce cerebral edema, even when administered up to 24 hours after the experimental injury. Mechanistically, the protective effects of progesterone may be attributed to its ability to reduce complement factor C3, glial fibrillary acidic protein (GFAP), and nuclear factor kappa beta (NFκB) in the TBI model (25), and decrease the levels of lipid peroxidation (26).

And in animal models that simulate demyelinating disease, progesterone has also been found to have significant neuroprotective potential, as evidenced by its ability to increase the expression of myelin proteins in the damaged sciatic nerves of young adult rats with nerve crush injuries (27). Furthermore, progesterone has also been shown to promote morphological and functional recovery in the Wobbler mouse, an animal model of spinal cord degeneration (28, 29).

These pieces of evidence that were obtained from animal models certainly supported the neuroprotective potential of these steroid hormones, and offered some insight into the mechanisms by which these hormones may be exerting their protective effects.

However, when this neuroprotective potential of estrogens and progesterone was applied to certain clinical trials, such as the WHIMS, the results were far from expected and were in fact, deemed inconsistent with the data derived from various animal studies. The estrogen formulation, Premarin (30, 31) or the combined estrogen/progestin formulation, PremPro, increased the risk for dementia and stroke (32-34) unlike what the animal studies would have predicted. And though the temptation has been to place blame on either the basic

science data or alternatively, the clinical studies, we argue that the data resulting from animal models, were on the contrary, consistent with the WHI studies and arguably, predicted the results of the WHI.

### 4. FACTORS THAT INFLUENCE HORMONE INDUCED NEUROPROTECTION

#### 4.1. The importance of age

The ovariectomized model is an excellent model to study the effect of estrogen and/or progesterone without the potential confound of having endogenous levels of estrogens and progesterone being contributed by the ovary. However, the ovariectomized animal best mimics the surgically menopausal woman, and may not necessarily model the menopause. Thus, the animal studies in which estradiol and/or progesterone administration to a young adult, ovariectomized animal has yielded beneficial effects, such as neuroprotection, should be interpreted as predicting the beneficial effects of a specific form of estrogen (17 $\beta$ -estradiol), and/or progesterone in younger, premenopausal women (perhaps those who have undergone bilateral oophorectomy). The WHIMS, however, assessed the effects of a particular estrogen formulation on older women who were on average 10 years past the menopause.

Animal studies have, in fact, demonstrated that (reproductive) age influences the protective effects of estradiol. For example, estradiol was found to be effective at protecting against scopolamine-induced cognitive impairment in rats that were beginning to go through the phase of reproductive senescence (as evidenced by a phase of constant estrus). However, in older animals (that were in a state of constant diestrus), estradiol treatment was ineffective (35). Moreover, Nordell and colleagues (36) have demonstrated that in young adults, estradiol administration to an ovariectomized rat attenuates the pro-inflammatory effects of an excitotoxic lesion, whereas estradiol exacerbated the toxicity in older, reproductive senescent animals. Thus, data from animal studies have suggested that neuroprotective efforts with estrogen in older animals is not effective and may actually exacerbate pathological cascades, and as such, are perfectly consistent with the WHI trial in which estrogen treatment of older women was found to lead to an increased risk for dementia and stroke. Furthermore, new reports suggest that, when the WHI data were segregated according to age, younger women were more likely to benefit from hormone therapy, whereas older women who were approximately 10 years beyond the menopause were prone to the negative consequences of hormones therapy (37).

#### 4.2. The type of hormone matters

Another important distinction between those animal studies in which estrogens and/or progesterone has been found to be protective and the clinical trials in which an estrogen or an estrogen and a progestin preparation had negative consequences lies in the choice of hormone used. Basic science studies have, in fact, demonstrated that progesterone is neuroprotective (19, 26, 28, 29, 38-40), while the synthetic progestin, MPA, is not (38, 39, 41). For example, in a model of stroke (reversible focal stroke using

the intraluminal filament model followed by 22 hours of reperfusion), MPA diminished the protective effects of conjugated equine estrogens (CEE) and MPA diminished estrogen's ability to reduce stroke damage (41). Interestingly, with regards to the traumatic brain injury model, MPA required a larger dose than P4 to accomplish a comparable reduction in cerebral edema. However regardless of the dose of MPA, MPA did not favor a better behavioral recovery than progesterone (reviewed in (42)).

Therefore, while P4 does not interfere with the beneficial effects of estrogens, MPA appears to have the capacity to prevent estrogen's beneficial effects. Such data requires us to consider the possibility that some of the negative consequences of hormone therapy observed in the WHI trials may have been a result of the choice of progestin used in the hormone therapy regimen.

#### 4.3. The delivery of estrogens and progesterone

In animal models, several routes of administration and delivery of steroid hormones have been used, including intravenous injection, subcutaneous injection, intranasal administration (43), oral gavage (44, 45), addition of the steroid to the water supply of animals (46), subcutaneous implantation of Silastic® pellets and implantation of the "matrix-driven delivery (MDD)" pellet (IRA, Sarasota, FL). The latter two methods have been used with the intention of providing continuous delivery of steroid hormones. While the use of Silastic® pellets have been demonstrated to be effective in delivering a constant level of 17 $\beta$ -estradiol (17 $\beta$ -E2) over weeks (10, 47) to months (3) in rats, the delivery of constant and sustained levels of hormone (either 17 $\beta$ -E2 or P4) to mice has proven to be more problematic. To obviate the problem of sustained steroid hormone delivery to mice, several approaches have been tested, including oral gavage, addition to the water supply of the animals (46) and implantation of the IRA MDD pellet system.

The later method of steroid delivery have become widely used, with IRA reporting 924 published papers using their 17 $\beta$ -E2 pellets and 142 reports using their P4 pellets ([www.innovrsch.com](http://www.innovrsch.com)). However, we have discovered that these pellets have not been validated for the kinetic of release of the steroid in mice. Instead, most published reports that utilized the 17 $\beta$ -E2 pellets from IRA cite either the IRA website ([www.innovrsch.com](http://www.innovrsch.com)) or other publications to support a continuous release of 17 $\beta$ -E2. Surprisingly, we found that these citations, in turn, lead to a single *ex vivo* dissolution study of one of the IRA pellets (48). Indeed, most studies assess levels of E2 at the termination of their study and claim continuous release of the steroid at the reported concentration throughout the duration of treatment. Our data, however, in which we assessed the acute (up to 7 days) release characteristics of these steroid pellets suggest that this is not a valid assumption, and instead, show that both 17 $\beta$ -E2 and P4 pellets produce a huge initial burst of hormone, followed by a gradual decline that even after 7 days is far exceed the target mid-estrous cycle target levels of 50 pg/ml for 17 $\beta$ -E2 and 4 ng/ml for P4. (Tables 1 and 2).

## Estrogens, progestins and neuroprotection

**Table 1.** Concentration of 17  $\beta$ -E2 at various times after implantation of IRA “matrix-driven delivery (MDD)” pellets containing 17  $\beta$ -E2

Hours after implantation	Serum 17 $\beta$ -E2 concentration (pg/ml)	Pellet 17 $\beta$ -E2 Content (mg)	Proposed duration of 17 $\beta$ -E2 release
1	13,044	0.25	60
4	10,563	0.25	60
8	2,536	0.25	60
12	1,113	0.25	60
24	965	0.25	60
Days after implantation			
4	972	0.25	60
7	856	0.25	60

17  $\beta$ -E2: 17 beta estradiol

**Table 2.** Measured serum progesterone levels at various times following implantation of the IRA “matrix-driven delivery (MDD)” progesterone pellets

Hours after implantation	Serum Progesterone concentration (ng/ml)	Pellet Progesterone Content (mg)	Proposed duration of Progesterone release
1	133.2	10	60
4	165.2	10	60
8	191.9	10	60
12	97.9	10	60
24	79.6	10	60
Days after implantation			
4	37.2	10	60
7	38.6	10	60

The results reported here could also explain the results recently reported by Green *et al.* (49), who used a similar treatment with a 0.25 mg, 90-day release 17 $\beta$ -E2 pellets from IRA. They observed that at the end of 90 days, plasma estradiol was elevated 3-fold over ovary-intact controls, uterine weights were 81% higher than ovary-intact controls and pituitary weights were 2.6-fold greater than ovary-intact controls. Additionally, two of the 17 $\beta$ -E2 treated animals had to be euthanized due to vaginal hyperplasia. These findings are consistent with what has been seen with high bolus doses of estrogens, which are known to cause permanent changes in rodent reproductive function (50, 51) including growth of uterine tissue in rodents (52). As such, the initial release of 17 $\beta$ -E2 following implantation could have contributed to the observed uterine, vaginal and pituitary hyperplasia. Additionally, the few studies using mice that have measured 17 $\beta$ -E2 concentrations as an end parameter have reported abnormal pharmacological responses (49) or strikingly high 17 $\beta$ -E2 concentrations (53, 54), even as long as 35 to 90 days after implantation of the pellets (55-58).

Most reports using IRA pellets sample for the levels of 17 $\beta$ -E2 or P4 at the termination of the study [for example, see (49, 55-58)], and only a few of these studies report levels of the ovarian hormones within or near physiologically relevant concentrations at that time (49, 57). Regrettably, many reports state their pellets produce physiological levels of 17 $\beta$ -E2 based upon technical information provided by IRA [for example see (59, 60)].

Based upon our observation of a large initial release of steroid hormones after implantation of these pellets, we urge caution in the interpretation of results obtained from studies that use of these hormone formulations. For example, Theodorsson and Theodorsson reported negative effects of estrogen, when delivered using the IRA pellet (1.5mg), in a transient middle cerebral artery

occlusion (MCAO) model of cerebral ischemia (61). This is in sharp contrast to numerous reports that support a benefit of estrogen treatment, where estrogen reduces the lesion size following experimental stroke (11, 16, 62, 63). We argue that this discrepancy was due to differences in the levels of estradiol to which the animal was exposed. The high concentrations may have desensitized the brain to the protective effects of estrogen (potentially through receptor downregulation). Thus, a more accurate conclusion that should have been made is that “high dose” estradiol is not beneficial in preventing damage associated with transient MCAO.

Thus, these animal studies point to the importance of delivering the appropriate levels of estrogen, and as such, question whether persistent delivery of a high dose of estrogen and/or progestin (as is done in most hormone therapy regimens) is the appropriate means of delivering a therapeutic or protective dose of hormone.

#### 4.4. Caveats associated with animal models

Transgenic knockout animals have been valuable in defining potential mechanisms by which estrogens exert their protective effects. However, these models are not without their limitations. For example, in determining the relevant estrogen receptor in the neuroprotective effects of estrogen against experimental stroke, some studies support the role of ER- $\alpha$  (64) while others implicate ER- $\beta$  in mediating estrogen-induced protection (65). For example, Dubal and colleagues described that the protective effects of low dose estrogen (resulting in plasma levels that are ~ 25 pg/ml) against experimentally-induced stroke was abolished in ER- $\alpha$  knockout (ER $\alpha$ KO) mice (10). However, due to the apparent loss of negative feedback regulation at the level of the hypothalamus, ER $\alpha$ KO mice are exposed to much higher levels of estrogen than their wild-type counterparts (66). As such, the “threshold” for the protective effects of estrogen may have been much higher. Consistent with this idea, administration of higher

concentrations of estradiol (~ 200 pg/ml) to ER $\alpha$ KO mice was effective at reducing infarct volume (67, 68). Thus, depending on the region of the brain, either ER- $\alpha$  or ER- $\beta$  may be involved in mediating estrogen's protective effects.

## 5. PERSPECTIVE

The studies described herein strongly support the value of animal models in testing the neuroprotective potential for estrogens and progestins. In fact, numerous *in vivo* paradigms have supported the ability of estradiol and progesterone to protect against a variety of insults. And though these models have several advantages relative to human clinical trials, at least with regards to the ability to manipulate and control experimental variables, it is imperative that we also recognize the caveats and limitations of each model. Only by recognizing such caveats will we be able to effectively translate these findings to human studies and later, implement them toward the development of novel hormone-based therapies for the menopause and the postmenopausal period, during which the risk for various disorders increases.

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