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Neuroprotection by Ovarian Hormones in Animal Models of Neurological Disease

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Ovarian hormones can protect against brain injury, neurodegeneration, and cognitive decline. Most attention has focused on estrogens and accumulating data demonstrate that estrogen seems to specifically protect cortical and hippocampal neurons from ischemic injury and from damage due to severe seizures. Although multiple studies demonstrate protection by estrogen, in only a few instances is the issue of *how* the steroid confers protection known. Here, we first review data evaluating the neuroprotective effects of estrogens, a selective estrogen receptor modulator (SERM), and estrogen receptor α- and β-selective ligands in animal models of focal and global ischemia. Using focal ischemia in ovariectomized ERαKO, ERβKO, and wild-type mice, we clearly established that the ERα subtype is the critical ER mediating neuroprotection in mouse focal ischemia. In rats and mice, the middle cerebral artery occlusion (MCAO) model was used to represent cerebrovascular stroke, while in gerbils the two-vessel occlusion model, representing global ischemia, was used. The gerbil global ischemia model was used to evaluate the neuroprotective effects of estrogen, SERMs, and ERα- and ERβ-selective compounds in the hippocampus. Analysis of neurogranin mRNA, a marker of viability of hippocampal neurons, with *in situ* hybridization, revealed that estrogen treatment protected the dorsal CA1 regions not only when administered before, but also when given 1 h after occlusion. Estrogen rarely is secreted alone and studies of neuroprotection have been less extensive for a second key ovarian hormone progesterone. In the second half of this review, we present data on neuroprotection by estrogen and progesterone in animal model of epilepsy followed by exploration into ovarian steroid effects on neuronal damage in models of multiple sclerosis and traumatic brain injury.

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Introduction

Although the ovarian hormones, estrogen and progesterone, primarily regulate reproductive functions in the brain and periphery, they also influence the development, growth, differentiation, maturation, and function of the peripheral and central nervous systems. Estrogen functions as a neurotrophic molecule that supports neuronal viability and, under certain conditions, prevents neuronal cell death. Recent evidence suggests that progesterone may also contribute to these events, albeit to a lesser extent. Estrogen may act via three mechanisms: (i) it may function as a factor that regulates gene transcription after binding to its receptor (estrogen receptor-α [ERα] or ERβ) and interacting with an estrogen response element(s) present in the promoter region of estrogen-regulated genes; (ii) estrogen may bind to ERα and/or ERβ and interact with proteins in the cell membrane or the cytoplasm to activate second messenger systems, and (iii) it may act via receptor independent mechanism(s) as a free-radical scavenger. Progesterone's actions too have been ascribed to (i) transcriptional mechanisms via progestin receptors (PR), (ii) rapid signaling events, (iii) binding of progesterone metabolites to GABA receptors, or (iv) antioxidant actions much like those of estrogen. In viewing hormonal influences on models of neuronal injury, it is critical to appreciate that steroidal actions can be exerted on multiple processes that will ultimately affect whether a neuron lives or dies (Fig. 1), and that these processes have defined temporal patterns (Fig. 2). Thus, when the steroid is administered it will determine on which process it acts and whether or not treatment will be successful.

A large body of evidence indicates that estrogen protects against brain injury, neurodegeneration associated with Alzheimer's disease (AD) and Parkinson's disease (PD), as well as aging-related cognitive decline (for reviews see refs. *1–3*). Recent evidence further implicates estrogen as a neuroprotective factor against neuronal damage from epilepsy *(4,5)*, despite its reputation as a proconvulsant *(6–11)*, and against cytokine damage in animal models of multiple scler-

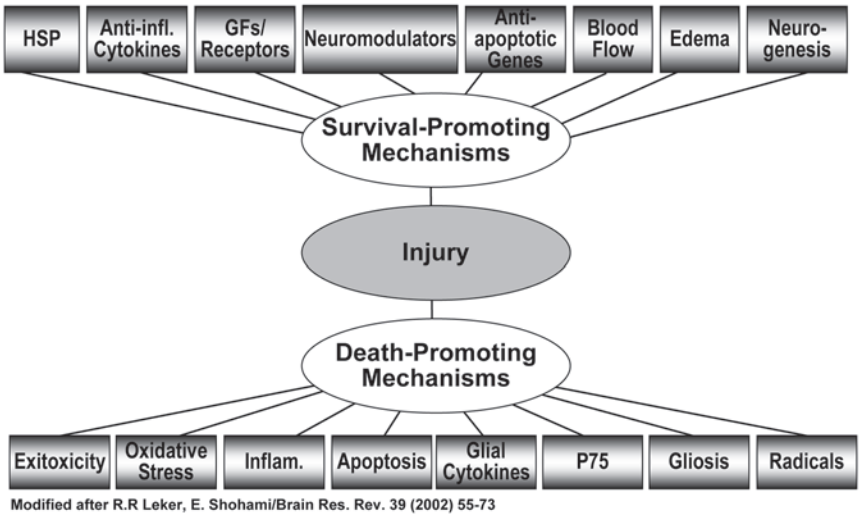

Fig. 1. Schematic showing target events by which steroids could either protect or enhance neuron cell death.

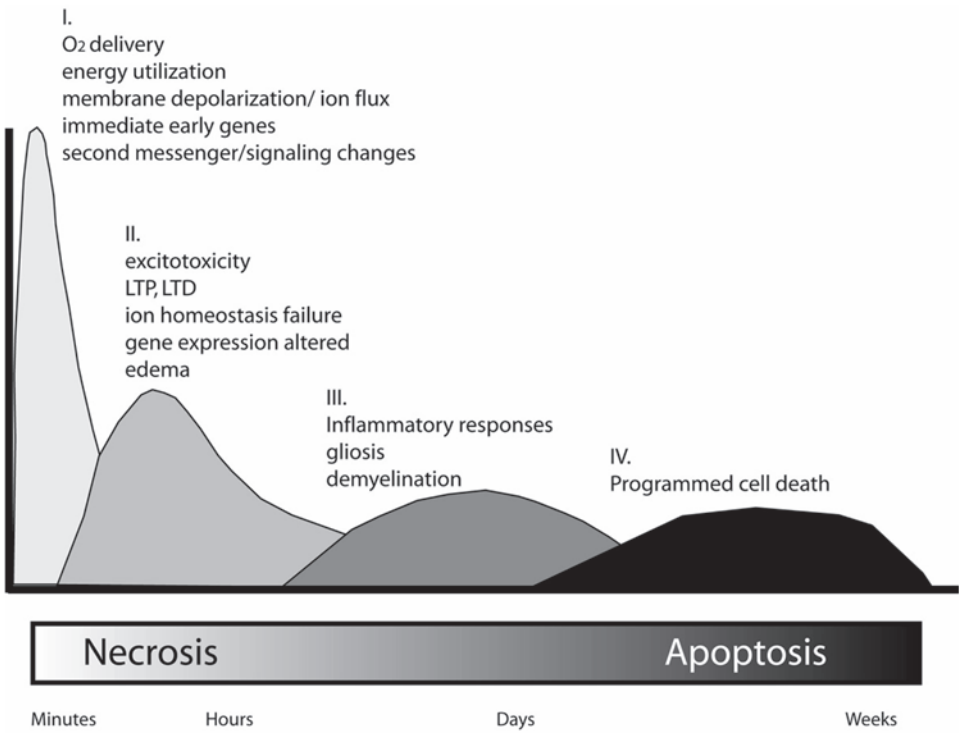

Fig. 2. Temporal sequence of events leading from initial injury to neuron death, any one of which could be influenced by ovarian hormones.

osis. Progesterone is used clinically to suppress seizure activity and to reduce edema following head injury; experimentally only low doses of progesterone are effective. Unfortunately, the mechanism by which the steroids mediate these effects is still uncertain. Understanding how these steroids act in brain injury becomes crucial as an increasing segment of the female population will spend a significant proportion of their lifespan in a hypoestrogenic, hypoprogesteronemic postmenopausal state. Unraveling the cellular and molecular mechanisms that underlie the protective actions of steroidal hormones may lead to new therapies for disorders/dysfunctions associated with loss of ovarian steroids.

In this review, we describe the use of a variety of animal paradigms that model stroke, epilepsy, autoimmune conditions similar to multiple sclerosis, and brain trauma. Through the use of these models and the investigation of different steroidal compounds, distinctly different roles for ovarian hormones in neurological diseases are emerging: estrogen is a potent neuroprotective agent that likely acts via both receptor-mediated and receptor-independent mechanisms to protect neurons from injury; whereas progesterone can dampen neuronal excitability but may have complex and dose-dependent interactions with molecules provoking cell death and processes affecting inflammation.

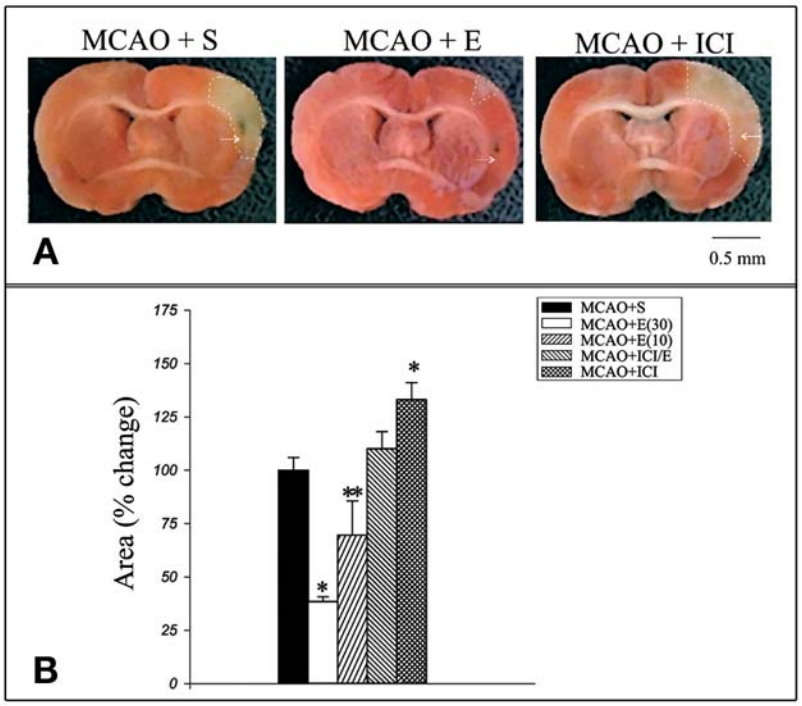

Fig. 3. (**A**) Digital photomicrographs illustrating representative examples of the extent of the infarct size within the insular cortex after MCAO and either saline, estrogen (30 min prior), or the ER antagonist ICI-182,780 (ICI) injections into the insular cortex. Infarct zones are outlined in each photomicrograph, and arrow indicates tip of injection cannulas in the region of the insular cortex. (**B**) Percentage change in infarct size relative to MCAO and saline (MCAO + S; 100%) in animals receiving either estrogen 30 min before [MCAO + E(30)] or 10 min before [MCAO + E(10)], estrogen and ICI-182,780 10 min before (MCAO + ICI/E), or ICI-182,780 alone (MCAO + ICI) injected 10 min before MCAO. *Significantly different from MCAO + S group; **significantly different from both MCAO + S and MCAO + E(30) groups (ANOVA; p , 0.05). Reproduced with permission from Saleh, T., et al. (2001). *Am. J. Phys. Integ. Comp. Physiol.* **281,** R2088–2095.

Estrogen Protects Against Permanent Focal Cerebral Ischemia

The animal models of permanent and transient focal ischemia were developed to model human cerebrovascular stroke *(12)*. Most often, a surgical nylon filament is inserted into the carotid artery and advanced to the circle of Willis at the base of the brain where it blocks blood flow into the middle cerebral artery (MCA). In the permanent ischemic model, the filament is maintained until euthanasia (typically 24–48 h), whereas in the transient model the filament is withdrawn 1–2 h after placement to allow reperfusion of the hypoxic brain region. While these treatment paradigms are dramatically different in the extent and severity of lesion, they model a range of human cerebrovascular strokes and thereby provide suitable conditions to study neuroprotective agents administered before (prevention), during, or after (post-treatment therapy) injury.

The administration of physiological *(13–16)* or pharmacological *(17)* levels of 17β-estradiol, prior to permanent *(15,18)* or transient *(13)* occlusion of the MCA, has been shown to dramatically reduce infarct volume as compared to vehicle-treated control rodents. Moreover, studies using a co-administration of estrogen and an estrogen receptor antagonist (ICI) revealed that the protection is ER-mediated (Fig. 3). Interestingly, the neuroprotective effect of estrogen in these models is confined to the cerebral cortex, with no detectable benefit seen in the striatum. Although in most studies estrogen was given for several days to a week prior to ischemic insult, recent studies have indicated that estrogen when administered as late as 3 h after injury *(19)* may still offer protection. Together, these data clearly illustrate that estrogen has a broad window of efficacy in models of stroke.

Does Estrogen-Mediated Neuroprotection Require ERs (ERα and/or ERβ)?

Additional studies confirmed that estrogen abated the ischemia-induced lesion in the cerebral cortex of rodents and revealed that ERα was dramatically and selectively upregulated in the injured, but not contralateral, cerebral cortex within hours after an ischemic event *(15,20)*. In contrast, the number of ERβ mRNA-expressing cells was reduced in the ipsilateral cortex, as compared with the contralateral side. Estrogen pretreatment did not affect the increase in cortical ERα expression, but did prevent the drop in ERβ-expressing cells, making it uncertain if estrogen's effects were due to ERα or ERβ. Subsequent studies utilized transgenic mice that had one of the ER isoforms "knocked out" (ERα KO and ERβKO) to assess the importance of each receptor.

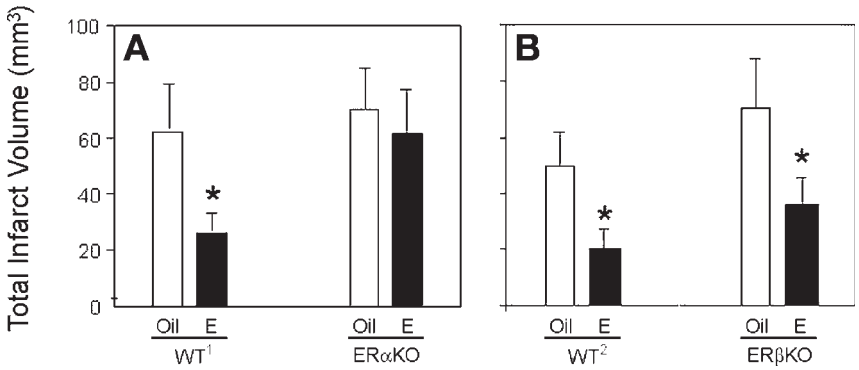

Fig. 4. Estrogen protects from focal ischemic injury as indicated by the total infarct volume in wild-type (WT) mice of both genetic backgrounds (C57BL6J-129 and 129Sv) and ERβKO mice but not in ERαKO mice indicating the importance of ERα in mediating the neuroprotective action of estrogen. Reproduced with permission from Dubal et al. (2001). *Proc. Natl. Acad. Sci. USA* **98** (4), 1952–1957.

The results of these studies demonstrated that ERα, and not ERβ, was the critical ER responsible for estrogen-mediated neuroprotection in the rodent cerebral cortex (Fig. 4) *(18)*.

The marked increase in ERα levels in the ipsilateral cortex of rats and mice after ischemic lesion was quite remarkable because ERα is largely absent in an uninjured adult cortex. Estrogen receptors are abundant in the neonatal rat cortex through the first week of life and then decrease with age until adulthood *(21)*. The finding that there is a "reappearance" of ERα in the cerebral cortex after injury suggests that the cortex may undergo a dedifferentiation process, which may be critical for neural repair. Therefore, it is plausible that the newly synthesized or upregulated ERα may require dedifferentiation to produce neural repair (e.g., neurogenesis to replace lost neurons, attenuation of gliosis and/or inflammation).

Estrogen Modulates Cortical Gene Expression After Ischemic Injury

It is well established that cortical cells can die via either necrotic or apoptotic pathways after an ischemic event, depending on the size of the infarct and length of time until reperfusion. Estimates suggest that up to 50% of the cellular death is due to apoptosis *(22)*, with both extra- and intracellular signals reported to convey this process. Because apoptosis, in contrast to necrosis, can be reversible, therapeutic interventions have been designed to stop and/or reverse the apoptotic process and thus rescue neurons from cell death. The mechanisms leading to apoptotic cell death may include NFκB-dependent pathways, p53-dependent pathways, and/or the activation of inducible pro-apoptotic members of the *bcl-2* family (i.e., *bad* and *bax*). These apoptotic pathways then activate caspases, including caspase 3, ultimately resulting in DNA laddering and cell death (reviewed in ref. *23*). While the exact role of estrogen in these processes is not fully understood, studies have shown that estradiol can promote cell survival by inducing *bcl-2 (24)*, a survival factor that can block both necrotic and apoptotic cell death *(25–27)*. Because *bcl-2* acts upstream to prevent the activation of caspases, inhibits free radical formation, regulates calcium sequestration, and blocks the pro-apoptotic actions of other members of the *bcl-2* family, including *bax* and *bad (27)*, the ability of estradiol to induce *bcl-2* could have a profound impact on the extent of apoptotic death present after injury. In addition to inducing the expression of *bcl-2*, estrogen might also act to enhance antioxidant mechanisms, remyelinization, synaptogenesis, promote trophic factor production, as well as reduce excitotoxicity, glutamate receptor activity, and inflammation *(27)*.

Estrogen Protects Hippocampal Neurons After Global Ischemia

The Mongolian gerbil has a gene mutation that results in an aberrant vasculature network in the base of the brain (i.e., incomplete circle of Willis). Because of this alteration, transient ligation of the common carotid arteries (the two-vessel occlusion model) results in severe global ischemia, a model that mimics human hypoxia seen after cardiac arrest or cardiac surgery. When the common carotid arteries are ligated for 5 min and reperfusion is permitted, ischemia leads to the selective loss of the pyramidal neurons in the hippocampal CA1 region *(28)*. As the length of ischemia is extended, the number of affected brain regions progressively increases. The loss of neurons appears to involve two waves of death, an initial loss seen hours after ischemia due to necrosis, followed by a second wave that peaks 4–5 d later and is due to apoptotic mechanisms *(26,29)*. Studies using this model of global ischemia show that pretreatment of ovariectomized females with physiological or pharmacological doses of estradiol can prevent the loss of CA1 neurons (Fig. 5) *(30)*. Moreover, in vivo binding studies with ^{125}I-estrogen determined that gerbil CA1 neurons contain nuclear ERs, although the receptor subtype has not been determined. These initial observations suggested that, in gerbils, the neuroprotective effects of estrogen were the result of a direct, ER-mediated event.

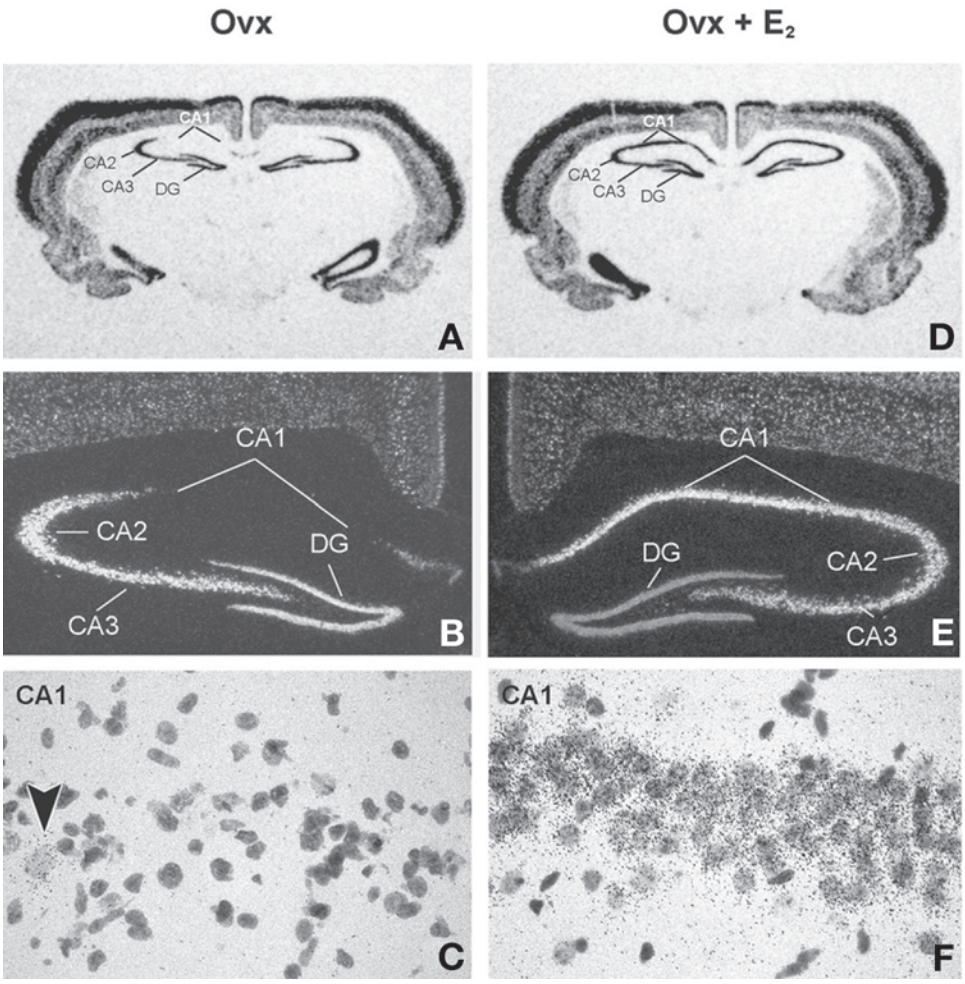

Fig. 5. Film and slide autoradiograms (dark and bright field) of neurogranin mRNA in the gerbil hippocampus by *in situ* hybridization. Note the dramatic and selective loss of neurogranin hybridization signal in the CA1 region of placebo-treated animals after injury (**A, B,C**). In contrast, neurogranin mRNA is still seen in the CA1 region of ovariectomized gerbils treated with 17β-estradiol (**D,E,F**). Reproduced with permission from Shughrue et al. (2003). *Neuroscience* **116,** 851–861.

Which ER Is Involved in Neuroprotection Following Global Ischemia in Gerbils?

Subsequent studies using ERα-selective and ERβ-selective compounds in gerbils revealed that both ERs are involved in hippocampal neuroprotection after stroke. The finding that estradiol was more efficacious than either ER-selective compound alone further indicated that both ERs participate and that each plays a unique role (Fig. 6A). In this regard, the global ischemia model may differ from that using middle cerebral artery occlusion. We have also found that steroidal and non-steroidal estrogens, such as estrone and diethylstilbestrol (DES), provide neuroprotection, while tamoxifen and raloxifene, compounds with partial estrogen receptor agonist/antagonist properties, were not effective *(30)* (Fig. 6B). In fact, tamoxifen and raloxifene were both capable of antagonizing the neuroprotective effects seen with estradiol.

Perhaps no ER is needed for estrogen to exert neuroprotective effects. Compounds with a phenolic A ring, including 17β-estradiol, tamoxifen, DES, and estrone, may act as antioxidants to prevent cell death *(1,31)*. Therefore, 17α-estradiol (a weak estrogen with a phenolic A ring) and vitamin E (an antioxidant that is not estrogenic) were evaluated in the gerbil ischemia model. Both 17α-estradiol and vitamin E offered some degree of protection *(30)*, an indication that estrogens can also act to protect neurons via non-receptor mediated mechanisms, including antioxidant pathways. However, the finding that tamoxifen and raloxifene, two non-steroidal antiestrogens with a phenolic A ring were unable to protect the hippocampal neurons is at odds with this theory.

Does Estrogen Provide Neuroprotection When Administered After the Ischemic Lesion?

In an attempt to better understand the genomic vs non-genomic actions of estrogens in global ischemia, ovariectomized gerbils were administered estrogens 0.5, 1, 2, or 4 h after a transient ischemic insult. The results clearly demonstrated that estrogen provided complete protection when administered 30 min or 1 h after the insult. However, when

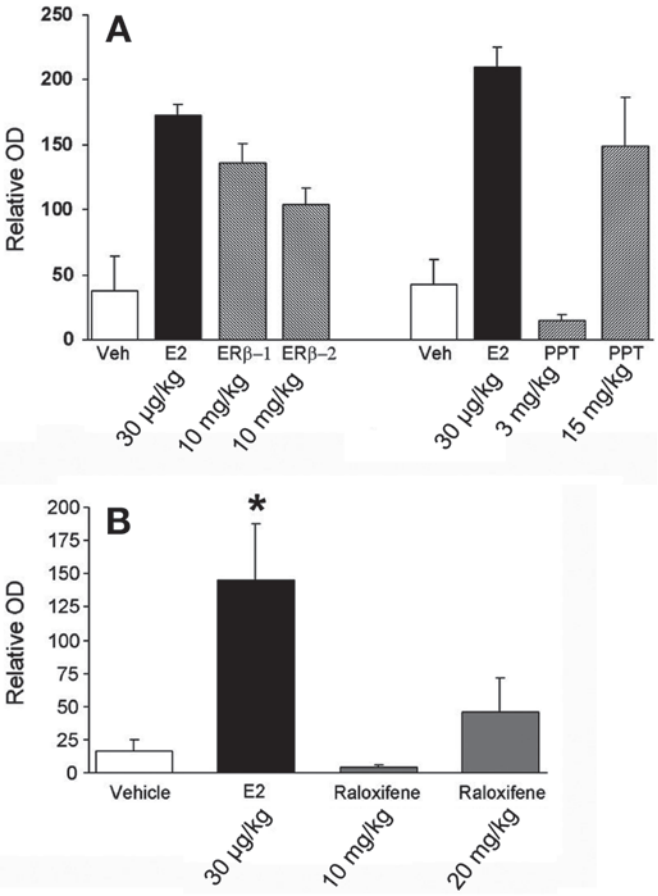

Fig. 6. (**A**) Analysis of film autoradiograms reveals that the level of neurogranin hybridization signal, as expressed as relative optical density, is very low in the CA1 region from placebo-treated ovx animals and high in animals treated with 17β-estradiol, the ERβ-selective compounds 1 and 2 (10 mg/kg, sc), and the ERα-selective compound (PPT). However, the ERα-selective compound provides protection only at 15 mg/kg and not at 3.0 mg/kg. (**B**) Similar analysis reveals that raloxifene at two different doses, 10 and 20 mg/kg, does not provide protection.

estrogen was injected 2 h after global ischemia, only partial protection was observed *(20)*. These findings are in agreement with a recent report which showed that estrogen reduced lesion size in rats when given up to 3 h after focal ischemia *(19)*. Rapid effects of estrogen have been ascribed to membrane receptors that produce changes in a variety of signaling cascades *(32)*. Based on the findings that estrogens can act rapidly to protect neurons *(33–41)*, one might speculate that estrogens act via a membrane-associated ER.

These conflicting data suggest that estrogens may act via several different mechanisms that are acting in concert to protect gerbil hippocampal neurons. The specific mechanism involved might depend on a variety of factors, including estrogen concentration, tissue of action, animal age, and the structure of the compound. At high, pharmacological concentrations, a variety of estrogen-like compounds appear to act as potent antioxidants, while at more physiological levels they are inactive as antioxidants *(1,42)*. In contrast, compounds such as 17β-estradiol are potent modulators of cell signaling pathways or transcription at physiological concentrations, acting via ER-dependent pathways. The localization of estrogen-binding sites in the gerbil CA1 neurons supports an action of estrogen at a cellular receptor, but whether that site is one of the classic nuclear ERs or a membrane receptor remains to be determined. Although the presented studies do not fully elucidate the mode by which estrogens protect the gerbil hippocampus, they do clearly demonstrate that a variety of steroidal and non-steroidal estrogens are potent neuroprotective agents in this animal model.

Intracellular Mechanism of Rapid, Membrane-Associated Estrogen Receptor–Mediated Neuroprotection

Studies aimed at dissecting out the intracellular events of neuroprotection by estrogen employed primarily neuronal cell lines or primary neurons of hippocampus and cortex either expressing the natural ERs or following transfection of the cells with ERα or ERβ cDNAs. A recent publication by Brinton's group *(43)* provides a summary of what we know today about the rapid signaling pathways participating in estrogen-mediated neuroprotection (Fig. 7). As shown, estrogen can activate multiple signaling cascades, including Src, extracellular signal-regulated kinase (ERK), phosphatidylinositol-3-kinase (PI3-K), protein kinase B (Akt), G protein–coupled signaling, c-fos and protein kinase C (PKC) that ultimately regulate the activation of multiple genes and subsequent neuronal function. According to the hypothesis summarized in Fig. 7, estrogen initiates a signaling cascade that is both dependent on and regulated by Ca^{2+}. Estrogen activates membrane-associated estrogen receptors, which in turn induce Ca^{2+} influx through activation of L-type Ca^{2+} channels. The initial rise in intracellular Ca^{2+} is required for estrogen activation of Src kinase either directly or through Ca^{2+}-dependent PKC. Scr then activates downstream ERK, which is subsequently translocated into the nucleus where it activates CREB, which, in turn, induces Bcl-e gene expression. Estrogen by increasing Bcl-2 expression not only promotes the expression of Bcl-2–regulated genes and subsequent neuroprotection but protects from Ca^{2+} overload by promoting Ca^{2+} sequestration into mitochondria without concomitant loss in mitochondrial viability. In addition, estrogen activates the Pi3-k/Akt pathway which leads to the inhibition of the bcl-2 antagonist BAD. The net result is elevated bcl-2 expression and subsequent neuroprotection. In addition to bcl-2, however, other genes may also be activated by CREB and participate in neuroprotection *(43)*.

Progesterone Protects Cortical Neurons from Focal Ischemia

It was shown almost a decade ago that progesterone was neuroprotective following experimental traumatic brain injury *(44)*. These observations have been confirmed by recent studies of Grossman et al. *(45)* showing that proges-

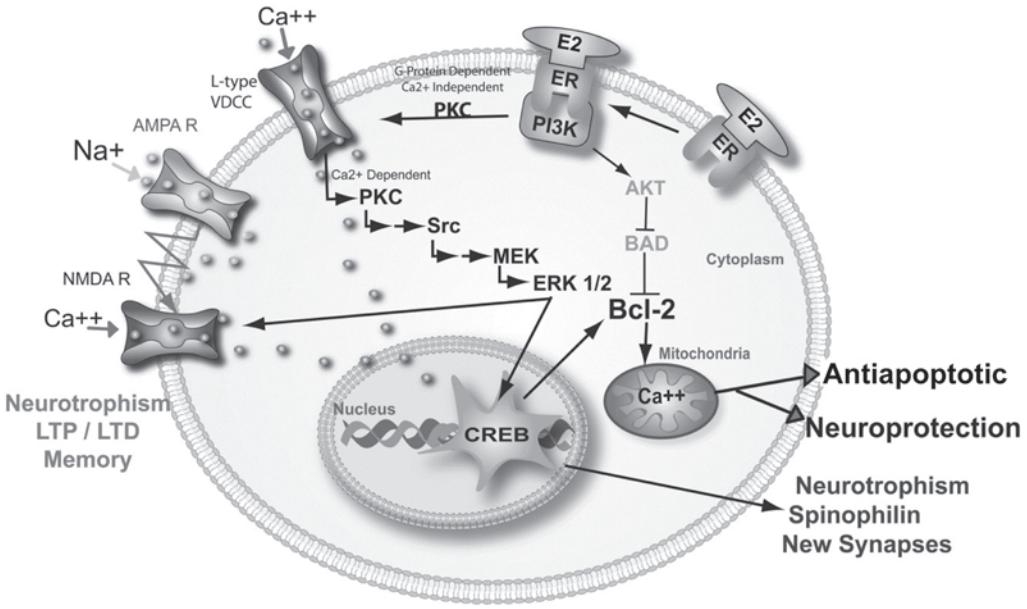

Fig. 7. Schematic representation of intracellular events leading to neuroprotection. Estrogen binds to membrane-associated receptors, which subsequently activates PI3K. PI3K then modulates L-type Ca^{2+} channels and induces Ca^{2+} influx. Elevated Ca^{2+} activates Src kinase, which, in turn, activates ERK and induces the translocation of the activated ERK into the nucleus. Here, ERK stimulates CREB and the activation of genes regulated by CREB, e.g., Bcl-2. Activated PI3K however, also activates Akt, leading to the inhibition of BAD, a Bcl-2 antagonist. Reproduced with permission from Wu et al. (2005). *Neuroscience* **135,** 59–72.

terone suppresses inflammatory response following traumatic brain injury *(45,46)* and brain edema even when administered 24 h after the injury *(47)*. Recent studies also indicate that progesterone is neuroprotective in spinal cord injury *(48)*, in transient focal ischemia *(49–51)* and permanent focal ischemia *(52)*. In addition to reducing edema, the progesterone-mediated neuroprotection involves the downregulation of pro-inflammatory cytokines (e.g., IL-1β, TNF-α, TGF-β2) *(53)* and NOS-2 *(52)*. It is believed that the action of progesterone on IL-1β expression is indirect via the regulation of the expression of NOS-2 *(52)*. This notion is supported by the observations that downregulation of IL-1β is not present in NOS-2 –/– mice *(52)* suggesting that a functional NOS-2 gene is required for progesterone to have neuroprotective effects acting either directly or indirectly via suppressing IL-1β expression. In addition to these actions via classic nuclear progesterone receptors (PRs) *(54–56)*, the neuroprotective effect of progesterone also involves rapid, membrane receptor–mediated mechanism, and progesterone may also function as a free-radical scavanger. A rapid action of progesterone involves an action on ion transport whereby progesterone may alter sodium transport from blood to brain via Na, K-ATPase and thus, reduce brain edema *(57,58)*. It is important to note, however, that progesterone's effects may change from protective to deleterious when plasma levels are elevated to the pharmacological range *(49)*. Aspects of the effect of progesterone's dose on protection will be discussed below.

Estrogen and Progesterone Protect Hippocampal Neurons After Epileptic Seizures but by Different Mechanisms

In women, the pattern of complex partial seizures (involving the limbic system) is influenced by the hormonal changes that occur across the menstrual cycle *(8–10,59–61)*. Increased seizure incidence is observed in the menstrual phase, when both estrogen and progesterone levels are low, as well as in the follicular phase, when estrogen levels are on the rise. By contrast, decreased seizure incidence is noted during the luteal phase when progesterone levels are high relative to estrogen. In animals, estrogen administration decreases while progesterone increases seizure thresholds *(62–69)*; these differential steroid effects are used to explain the cycle-dependent changes in seizure patterns in women. Indeed, the effects observed with progesterone form the basis for progesterone treatment of women with catemenial epilepsy *(9,10,60,61,63,70–72)*.

Limbic system seizures, when persistent, increase the risk of permanent damage to the hippocampal formation in the form of hippocampal sclerosis *(73–77)*. In animals, the use of the toxin kainic acid (KA, an excitatory amino acid analog) produces limbic seizures that damage neurons in the hippocampal formation and surrounding structures, particularly CA1, CA3, hilus, and piriform/entorhinal cortex, while sparing CA2 and the dentate gyrus *(62,78–83)*. Progesterone treatment reduces limbic seizures in a variety of experimental models *(66,84,85)* but in vivo has no neuro-

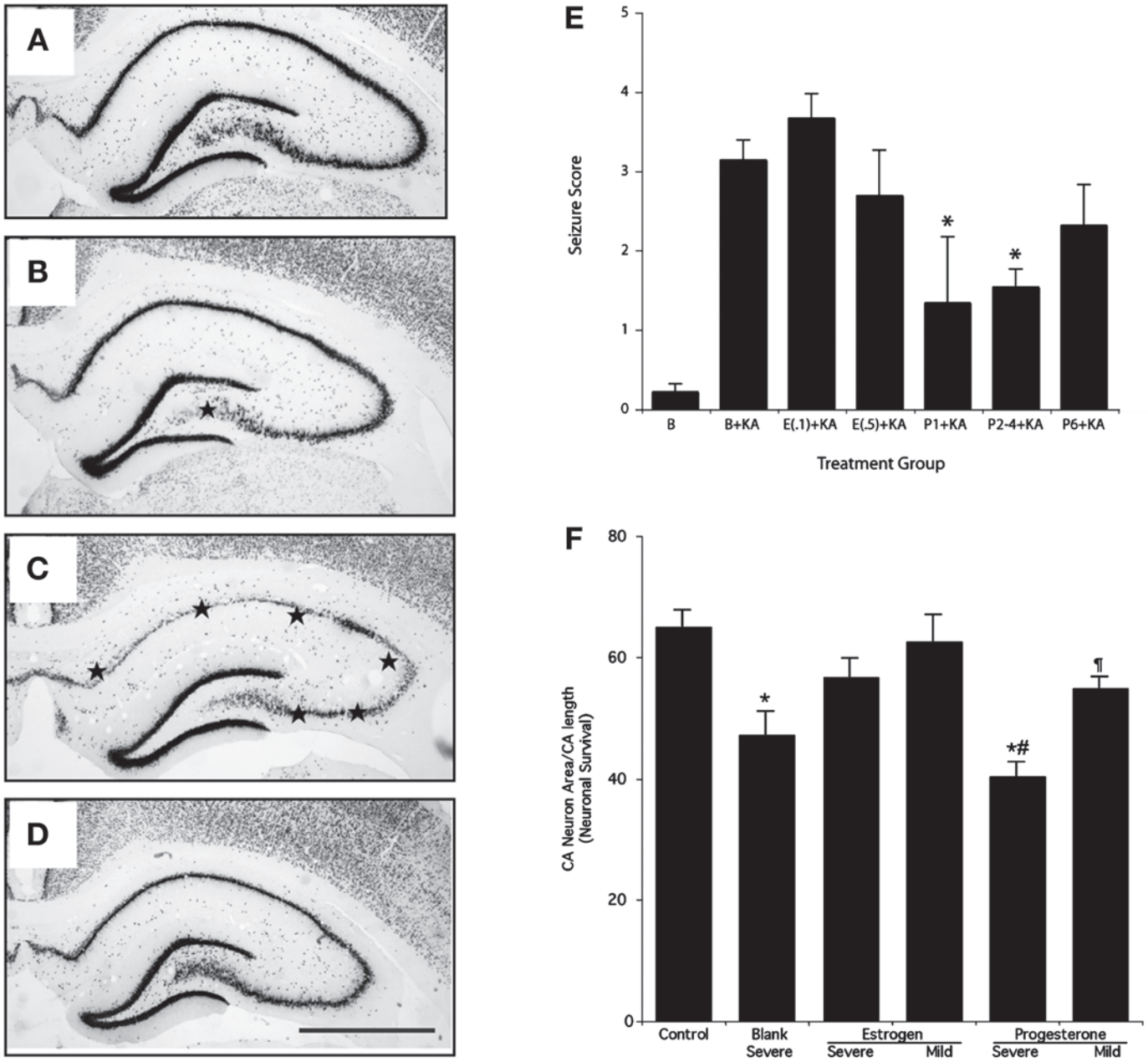

Fig. 8. Sections through the hippocampus of ovariectomized (ovx) female rats replaced with progesterone (**A–C**) or estrogen (**D**) for 1 wk and then treated with 8.5 mg/kg kainic acid. Animals were euthanized 72 h later and the brains were stained for neuron nuclear antigen. (**A**) Section from an ovx animal that received progesterone and exhibited low levels of seizure activity. (**B**) Section from an animal replaced with progesterone that exhibited moderate seizures after kainate. Note the presence of neuron loss in the hilus (star). (**C**) Section from an animal replaced with progesterone that exhibited severe seizures after kainate. Damage was extensive in CA1, CA3, and the hilus (stars), but CA2 and the dentate gyrus were spared. (**D**) Section from a rat that was replaced with estrogen and had severe seizures shows no apparent loss of neurons. Bar = 100 μm. (**E**) Bar graphs showing the effects of hormone treatments on seizure scores. Estrogen doses were either 0.1 or 0.5 μg/d. One, two, four, or six capsules of progesterone were used; each progesterone capsule delivered approximately 10 ng/mL. Note that low, but not high, levels of progesterone suppressed seizure activity, whereas estrogen did not affect the seizure scores produced by kainate treatment. (**F**) Bar graphs showing the immunoreactivity for neuron nuclear antigen (surviving neurons). Note that estrogen reduced loss of neurons from severe kainate seizures. Reproduced with permission from Hoffman et al. (2003) *(4)*.

protective effects of its own apart from its effects on seizure activity *per se (4)*. This feature is illustrated in Figs. 8A–D. In Fig. 8A is shown the hippocampus of a control animal. When ovariectomized animals are treated with progesterone and show suppressed seizures (Fig. 8B), only minimal damage to the hippocampus is seen. If seizure activity breaks through the progesterone blockade, then marked damage to the hippocampus is noted and this degree of damage is equiv-

alent to animals that did not receive progesterone but had equally severe seizures (Fig. 8C). Moreover, the ability of progesterone to suppress seizures appears to be dose-dependent: low but not high physiological levels of progesterone suppressed seizures and reduced hippocampal damage (Figs. 8E and 6F) *(4)*.

One possible mechanism for the seemingly contradictory actions of progesterone at high and low doses may lie in the steroid's ability to modulate the GABAA receptor. Progesterone is metabolized to 3-alpha-hydroxy-5-alpha-pregnan-20-one (allopregnanolone), a potent allosteric modulator of the $GABA_A$ receptor *(86)*. Several studies have suggested allopregnanolone, acting at the $GABA_A$ receptor, is the mechanism whereby progesterone attenuates seizure activity *(61, 62,87–90)*. For example, Frye *(87)* reported that subcutaneous administration of allopregnanolone 3 h prior to perforant path stimulation significantly reduced both seizure severity and the resulting hippocampal neuronal loss. Levels of allopregnanolone and $GABA_A$ receptor activity in vitro are positively correlated with progesterone levels *(91)*, raising the expectation that in vivo increases in plasma progesterone should result in increased seizure suppression.

In vitro, prolonged exposure of cortical neurons to allopregnanolone abolishes the potentiation of $GABA_A$ receptors by altering the allosteric interactions of allopregnanolone with the benzodiazepine binding sites *(92)*. In vivo prolonged treatment with either progesterone or allopregnalonone produces desensitization and it was proposed that alterations in $GABA_A$ receptor subunit expression were responsible *(93,94)*. If this phenomenon is dose-dependent, such changes in the $GABA_A$ receptor composition could explain why low but not high doses of progesterone reduced seizures after KA.

For estrogen, initial studies suggested that despite the potential for increased seizures, estrogen may reduce neuronal death from seizures *(5,95)*. However, those studies only used injected steroid (which produces variable hormone levels) and doses that often exceeded the physiological range. More recent studies using low doses of kainic acid to induce seizures determined that estrogen has little beneficial effect on seizure severity (Fig. 8E) but reduces mortality from seizures and is capable of protecting the hippocampus from seizure damage *(4)* (Figs. 8D and 8F).

How estrogen protected the hippocampus from seizure-induced damage is not immediately clear. Initially, KA cell death was thought to be exclusively necrotic and so there would be little basis for estrogen interfering in that process. More recent studies demonstrate that delayed cell death accompanied by DNA laddering (normally associated with apoptosis) also occurs after KA-induced seizures *(96–102)*. The pro-apoptotic molecule Bax is upregulated following KA seizures and concomitantly the pro-survival molecule Bcl-2 is downregulated *(103)*. Because estrogen upregulates expression of Bcl-2 in a variety of models *(15,104–107)*, this mechanism could explain estrogen's protective effects after seizures. Alternatively, because positive effects of estrogen were seen when plasma levels were high enough to produce antioxidant effects, protection from free radicals could be invoked to explain the estrogen effect. However, high progesterone levels should also have been antioxidant *(108)*, but in fact did nothing to protect the animals from seizures or their damage *(4)*, as was discussed above.

Neuroprotection in Traumatic Brain Injury

Trauma to the brain produces edema, primary death to neurons at the site of impact, and secondary neuronal damage to underlying areas. Focus on the role of gonadal steroids in trauma-induced brain damage began with the observation that following traumatic insult, females showed less edema than males *(47)* and had reduced cortical contusions compared with males *(109)*. Focus then centered around progesterone because in states of hyperprogesteronemia in females, edema was virtually absent (47). Subsequent studies showed effects of progesterone in males and examined both edema and cognitive recovery *(110)*. It was logical that the reduction in edema would reduce the likelihood of secondary damage to the hippocampus normally seen in models of traumatic brain injury. Indeed, in a recent study of secondary injury to the hippocampus in ovariectomized female rats, progesterone at low but not high physiological levels protected the CA1 and CA2 subfields from neuronal loss *(111)*.

How high levels overwhelm progesterone's protective actions is still unknown. The mechanisms whereby progesterone produces its effects in traumatic brain injury are not fully understood. Molecular changes suggest that some neuroprotection by progesterone can arise from reduced pro-apoptotic and inflammatory cytokine production *(112)* and lessened mitochondrial dysregulation *(111)*. A membrane-associated protein that binds progesterone (25-Dx) and is located on neurons that regulate water and ion homeostasis in the central nervous system *(113)* could contribute to progesterone's effects. Progesterone's effects on temperature regulation could also play a role in dictating neuronal survival. In females ovariectomy prior to traumatic brain injury enhances post-traumatic hyperthermia and this feature could contribute to neuronal survival after injury *(114)*. Whether any of these changes involve participation of progesterone receptors is unknown.

The role of estrogen in traumatic brain injury is less well studied but a role for estrogen's antioxidant properties has been proposed *(115)*.

Steroid Effects in Animal Models of Multiple Sclerosis (MS)

Neuroprotective effects of ovarian hormones have also been extended to studies that model multiple sclerosis (particularly experimental allergic encephalitis, EAE). Multiple sclerosis (MS) is an autoimmune disease of the nervous

system with inflammatory flare-ups that are initially intermittent. While MS patients can show complete recovery between episodes, with recurrence, neurological deficits eventually become irreversible, leading to cumulative disability. Axonal damage within demyelinated plaques in MS patients is reported to underlie severity of disease and increasing disability. Neuronal loss as a consequence of such damage in MS is proposed *(116)* and a recent report documented a significant increase in apoptotic neurons in demyelinated cortex of MS brains compared to normal, myelinated cortex *(117)*. An interaction of systemic factors in the MS disease process with neuron cell death is suggested by the ability of spinal fluid of MS patients to damage axons and induce apoptosis in cultured neurons *(118–120)*.

A consistent finding in MS and EAE is gender susceptibility, evidenced by high female:male ratios *(121)*. The sexual dimorphism likely reflects multiple factors including gender-specific hormonal effects on immune responsiveness, genetic susceptibility, and modulation of target cell activity. Ovarian hormones, through their ability to influence cytokine expression, may impact several different aspects of inflammatory disease. Estrogen, in particular, is thought to be beneficial in MS based on the fact that estrogen levels inversely correlate with the incidence of exacerbations, and that the disease is ameliorated during pregnancy and then is increased in the post-partum period *(122–124)*. Estrogen's ability to reduce flair-ups in MS is thought to work by skewing the T-cell cytokine profile from a Th1 to a Th2 response, limiting TNFα production, and limiting lymphocyte homing into the central nervous system *(125–131)*.

During pregnancy, a time when MS symptoms abate, both estrogen and progesterone are increased; however, progesterone has received relatively little attention in EAE. Although progesterone in vitro synergizes with estrogen to favor a Th2 profile *(132)*, it does not ameliorate EAE or enhance the estrogen effect on EAE in SJL mice (a strain sensitive to EAE demyelination) *(129)*. In a study of Lewis rats, progesterone administration surprisingly *increased* the severity of EAE and was associated with poorer recovery *(125)*. Additionally, physiological levels of progesterone, in the absence of estrogen, significantly increased the inflammatory infiltrates of EAE (Figs. 9A,B) as well as the disorder's clinical severity (Fig. 9B), and produced significant neuronal death *(133)* (Figs. 9C,D). While it is not known how progesterone produces these effects, they raise the possibility that progesterone increases the mononuclear infiltration and that process then predicts the disease severity. When estrogen and progesterone were combined, severity of EAE did not exclusively reflect the extent of the mononuclear infiltration *(133)* suggesting that estrogen was working on events that take place after the mononuclear cells have arrived in the CNS tissue. These observations suggest that estrogen exerts an additional benefit that limited CNS damage over and above its ability to limit inflammatory cell infiltration.

Steroid Actions in Neuronal Damage from Cytokine Exposure

Altered levels of the cytokine TNFα are thought to contribute to neuronal death in multiple neurologic diseases including multiple sclerosis *(134)*. One model used for study of neuronal responses to TNFα employs differentiated pheochromocytoma cells (PC12 cells). PC12 cells differentiated in the presence of nerve growth factor take on characteristics of neurons and became susceptible to TNFα-induced apoptotic death in a dose- and time-dependent manner. At physiologic concentrations of estrogen and progesterone, the PC12 cell death induced by TNFα was markedly reduced by estrogen, but significantly increased by progesterone *(135)*. These findings are consistent with observations of hormone effects (discussed above) using the rat EAE model *(133)*.

Are Steroid Receptors Mediating Estrogen's Neuroprotection and Progesterone's Exacerbation of Cytokine-Induced Damage?

Significant protection of PC12 cells by estradiol was a delayed event (Fig. 10A), requiring 18–24 h incubation preceding TNFα treatment *(135)*. Estrogen enhances neuron survival in a variety of models through specific receptor-mediated transcriptional regulation of genes encoding pro- and anti-apoptotic proteins, cytokines including TNFα, and cytokine receptors *(128–130,136–138)*. Following trophic factor withdrawal, estrogen-mediated survival of PC12 cells required ERα expression and survival was blocked by both transcriptional inhibitors and specific estrogen-receptor antagonists *(138)*. While specific ER involvement in estrogen's protection from cell death provoked by TNFα has not been tested, the increased PC12 cell survival produced by estrogen was accompanied by increased expression of estrogen receptor ERα and the anti-apoptotic Bcl-xL protein, while it decreased TNFR 1 expression (Figs. 10 C–E), consistent with an ERα-mediated mechanism.

Based on timing, rescue of PC12 cells from TNFα-induced apoptosis is less likely to be through estrogen membrane-receptor activation, because 24 h incubation with estrogen was required to increase significant cell survival. This same period of estrogen exposure was critically important for survival of cortical neurons to insult *(139)*; the mechanism of this effect required ERα. By analogy, the findings in PC12 cells treated with TNFα are consistent with ERα-mediated transcription of genes encoding proteins involved in various aspects of anti-apoptotic activity, although it is still possible that some signaling intermediate might accumulate over several hours in order to effect rescue through an ERα-independent mechanism.

Multiple mechanisms could contribute to progesterone's deleterious effects. Activity through nuclear progesterone receptors could explain the changes in TNFR1, Bcl-xL, and ERα expression associated with progesterone (Figs. 10C–E).

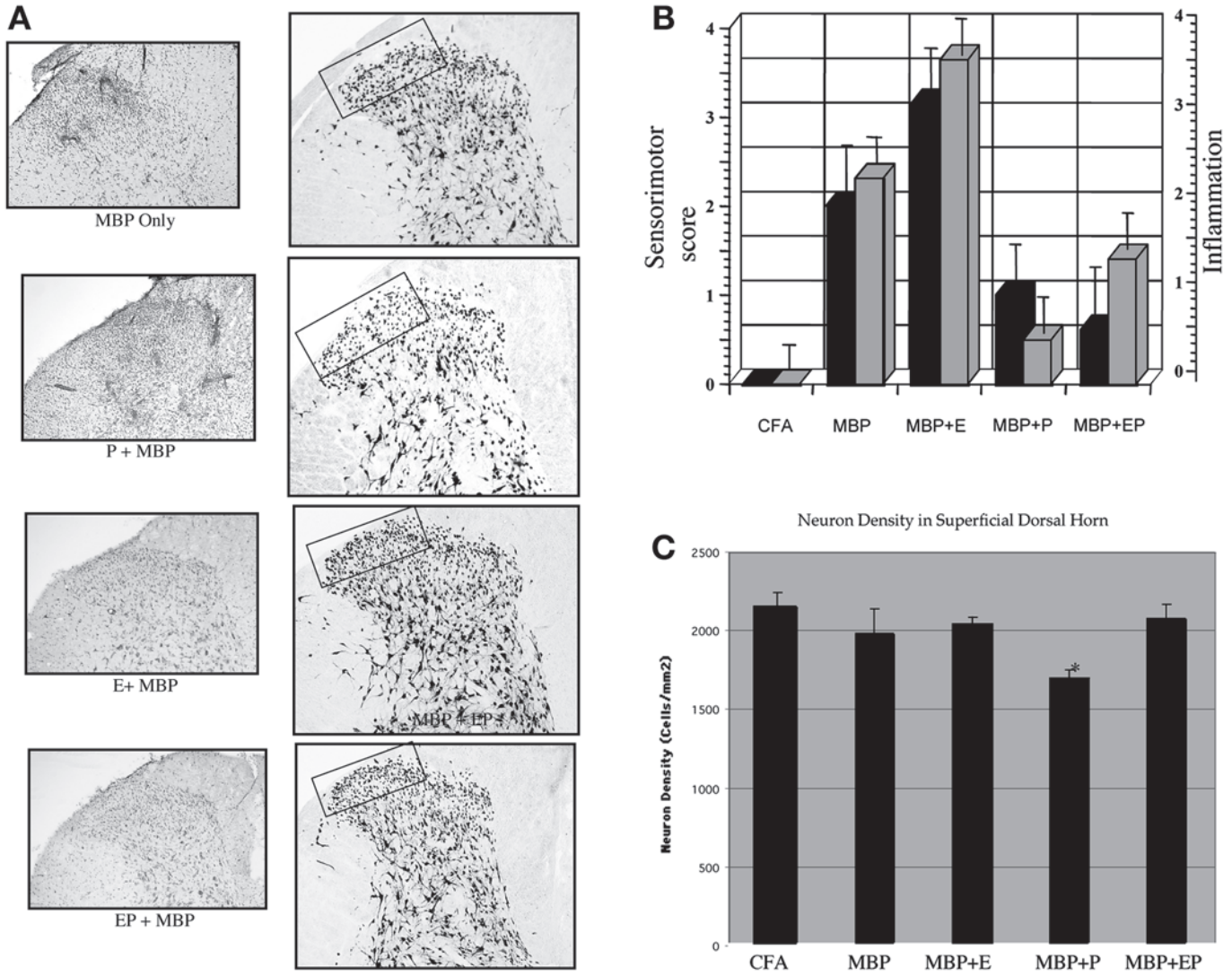

Fig. 9. (**A**) Sections of the dorsal horn of the spinal cord from ovariectomized female rats not replaced with hormones or replaced with estrogen, progesterone, or estrogen and progesterone that were immunized against myelin basic protein (MBP) to induce EAE and euthanized 11 days later. On the left are sections from the left dorsal horn stained with a Nissl stain (neutral red); on the right are adjacent sections from the same animals stained with Neuron Nuclear Antigen, a neuron-specific marker. The superficial dorsal horn is highlighted in the boxed area. Note that progesterone increased mononuclear infiltrates and that was associated with reduced numbers of neurons in the superficial dorsal horn. Estrogen either alone or with progesterone reduced infiltrates and these animals showed no neuronal loss. (**B**) Semiquantitative assessments of inflammation (gray bars) and sensorimotor scores (black bars) in EAE animals. Controls received only complete Freund's adjuvant (CFA) but no MBP. (**C**) Neuron densities in the superficial dorsal horn.

Pro-apoptotic activity accompanied by reduced Bcl-2/Bcl-xL expression was reported in an in vitro model of breast tissue using micromole levels of progestins *(140,141)* but was previously unrecognized in neurons.

Recently, a membrane receptor for progesterone was identified and was associated with rapid and nongenomic progesterone effects *(142)*. The detrimental effect of progesterone on neuronal survival induced in as little as 15–30 min (Fig. 10B) could reflect modulation of a downsteam pro-apoptotic signaling cascade through such a membrane receptor. TNFα activates downstream signaling molecules via its receptors, TNFR1 and TNFR2. Activation of the major receptor, TNFR1, leads to recruitment and activation of receptor–associated proteins TRADD, FADD, and the TNF receptor-associated factor 2, TRAF2 *(143)*. Activation of apoptotic pathway may be initiated by the binding and activation of caspase 8 to FADD. TNFα -induced apoptosis via TNFR1 also involves TRAF2-mediated activation of Rho GTPase, MEKK1, and JNK1 pathway *(143–145)*. This pathway is relevant to neuronal death, because differentiated neurons may undergo apoptosis through the JNK pathway *(116,145, 146)*. These two upstream signaling pathways involving caspase 8 and JNK are activated in PC12 cells exposed to TNFα *(135,147)* and appear to act in synergy at the level of mitochondrial damage (Fig. 11). Further study is needed to assess whether the changes in JNK activity and the observed

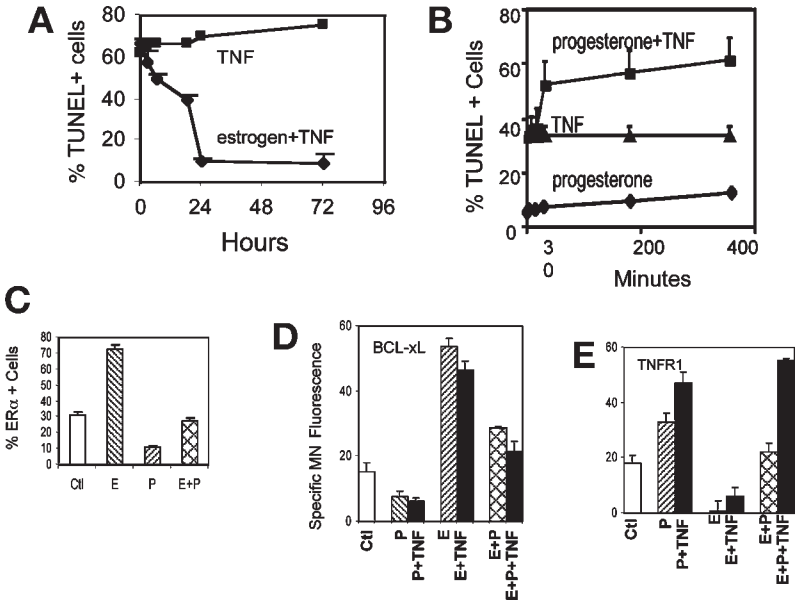

Fig. 10. Apoptosis in differentiated PC12 cells exposed to either estrogen (**A**) or progesterone (**B**) at various times prior to exposure to TNFα. Note that estrogen required approximately 14 h to maximally rescue cells from death, whereas the pro-apoptotic effects of progesterone were exterted rapidly. (**C**) Changes in ERα expression in PC12 cells exposed to TNFα. (**D**) Profiles of Bcl-xL expression in PC12 cells. (**E**) Profiles of TNFR1 expression in PC12 cells. Controls were differentiated but not exposed to TNFα.

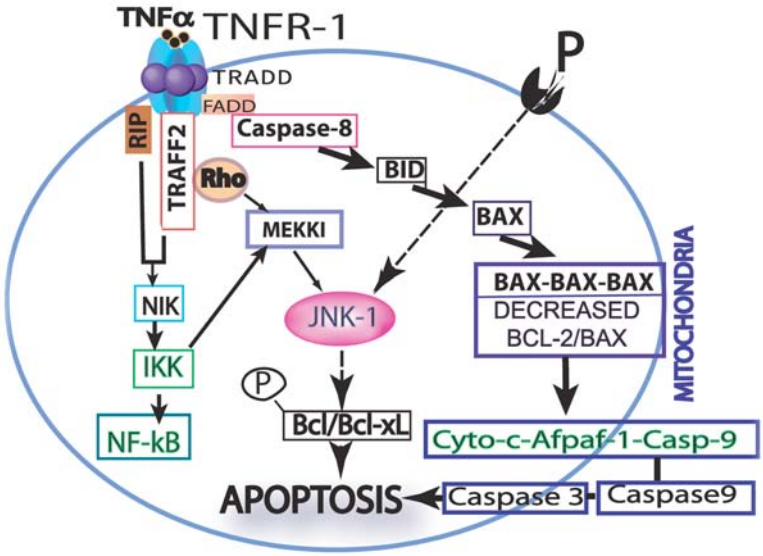

Fig. 11. Proposed pathway for progesterone's rapid effects on cell apoptosis.

changes in gene expression are independent. It is important to note that the two mechanisms are not mutually exclusive; rather, the rapid and delayed actions of progesterone could act in concert.

In summary, estrogens in diverse models of neurologic disease involving neuronal death exert neuroprotective actions probably via a large array of mechanisms including both genomic and nongenomic actions. The genomic actions include, among others, the stimulation of the expression of genes involved in survival-promoting mechanisms (e.g., *bcl*-family members, trophic factors, and their receptors) and inhibiting death-promoting mechanisms (e.g., p75, caspase-3, TNFα, IL-1 and IL-6), as well as possible rapid membrane ER-associated mechanisms. In addition, estrogens function as antioxidants and enhance antioxidant mechanisms, and they reduce excitoxicity and inflammation. Progesterone's effects in cell death are far more difficult to predict and largely depend on the dose of progesterone and on the type of insult. For diseases in which abnormal neuronal firing is present, progesterone can be metabolized to molecules that enhance GABA activity and are thus beneficial. In addition, low doses of progesterone reduce edema via mechanisms that are still unknown, providing improved outcome when trauma is present. Yet the

dark side of progesterone cannot be ignored, and high doses of progesterone are likely to negate its beneficial effects.

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