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Chronic but Not Acute Estradiol Treatment Protects Against the Neurodegenerative Effects of *N*-Methyl-D-Aspartate Receptor Antagonists

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Drugs that block NMDA receptors, thereby inducing an NMDA receptor hypofunctional (NRHypo) state, can cause a disseminated pattern of irreversible neurodegeneration. Based on several lines of evidence, an *N*-methyl-D-aspartate receptor hypofunction (NRHypo) mechanism has been postulated to contribute to neurodegenerative changes in Alzheimer disease (AD). Because estrogen putatively exerts a neuroprotective effect in AD, we examined whether estrogen protects against NRHypo-induced neurodegeneration. We administered estradiol benzoate in three separate experiments to adult female rats: (1) 100 µg subcutaneously as a onetime dose, (2) 100 µg bid twice daily for 4.5 or 14 d, and 3) 300 µg twice daily for 4.5 d. Two hours after the last estradiol dose, MK-801 was administered (0.5 mg/kg subcutaneously) to produce a robust neurotoxic injury. Controls received MK-801, but no estradiol. Four hours after administration of MK-801, the severity of injury was evaluated histologically by quantitative methods previously described. Compared to controls, a single dose of estradiol produced no change in the severity of injury ($p = 0.24$). Chronic treatment with estradiol was associated with a 25–35% reduction in the number of injured neurons ($p < 0.05$ in all cases). We conclude that chronic but not acute estradiol treatment reduces the severity of NRHypo-induced neurodegeneration.

Key Words: Estradiol; NMDA receptor antagonists; MK-801; neurodegeneration; neurotoxicity; Alzheimer disease.

Introduction

Glutamate (Glu) is the predominant excitatory transmitter in the mammalian central nervous system (CNS) and participates in many important functions in both the developing and adult brain. In addition, Glu in excessive amounts exerts an excitotoxic action that can injure or kill CNS neurons *(1)*. The excitotoxic actions of Glu are mediated by a family of ionotropic receptors, of which there are three subtypes, each named after an agonist to which it is differentially sensitive: *N*-methyl-D-aspartate (NMDA), amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainic acid *(2)*.

The excitotoxic action of Glu is responsible for neuronal degeneration in many acute neurologic disorders, including stroke, status epilepticus, hypoglycemia, CNS trauma, and certain types of food poisoning *(3–5)*. The NMDA Glu receptor subtype is widely distributed throughout the CNS and has been implicated in the mediation of many of these acute CNS injury syndromes. NMDA receptor antagonists have been shown to protect against neuronal degeneration in these various syndromes, but adverse CNS side effects, including psychotic reactions, have hampered the development of these drugs for human therapy.

In addition to the psychotomimetic properties of NMDA antagonist drugs, it was found, in the course of testing these drugs for their neuroprotective effects, that they can induce neurotoxic changes in cerebrocortical neurons *(6)*. Reversible changes are induced by low doses of these drugs and are detected primarily in neurons of the retrosplenial cortex (RSC) and, to a lesser extent, neurons of the entorhinal cortex; these changes consist of intracytoplasmic vacuoles, which have been characterized ultrastructurally as swollen mitochondria and dilated saccules of endoplasmic reticulum (Fig. 1B). Following higher doses, these same neurons, as well as neurons in several other corticolimbic brain regions, undergo irreversible degeneration (Fig. 1D; *[7,8]*). The mechanism underlying these neurotoxic changes has been traced to a complex network disturbance (Fig. 2), whereby blockade of NMDA receptors inactivates inhibitory GABAergic and noradrenergic neurons that normally hold major glutamatergic and cholinergic excitatory pathways under tonic inhibitory restraint. Disinhibition of these two excitatory pathways causes them to hyperactivate (through both non-NMDA Glu

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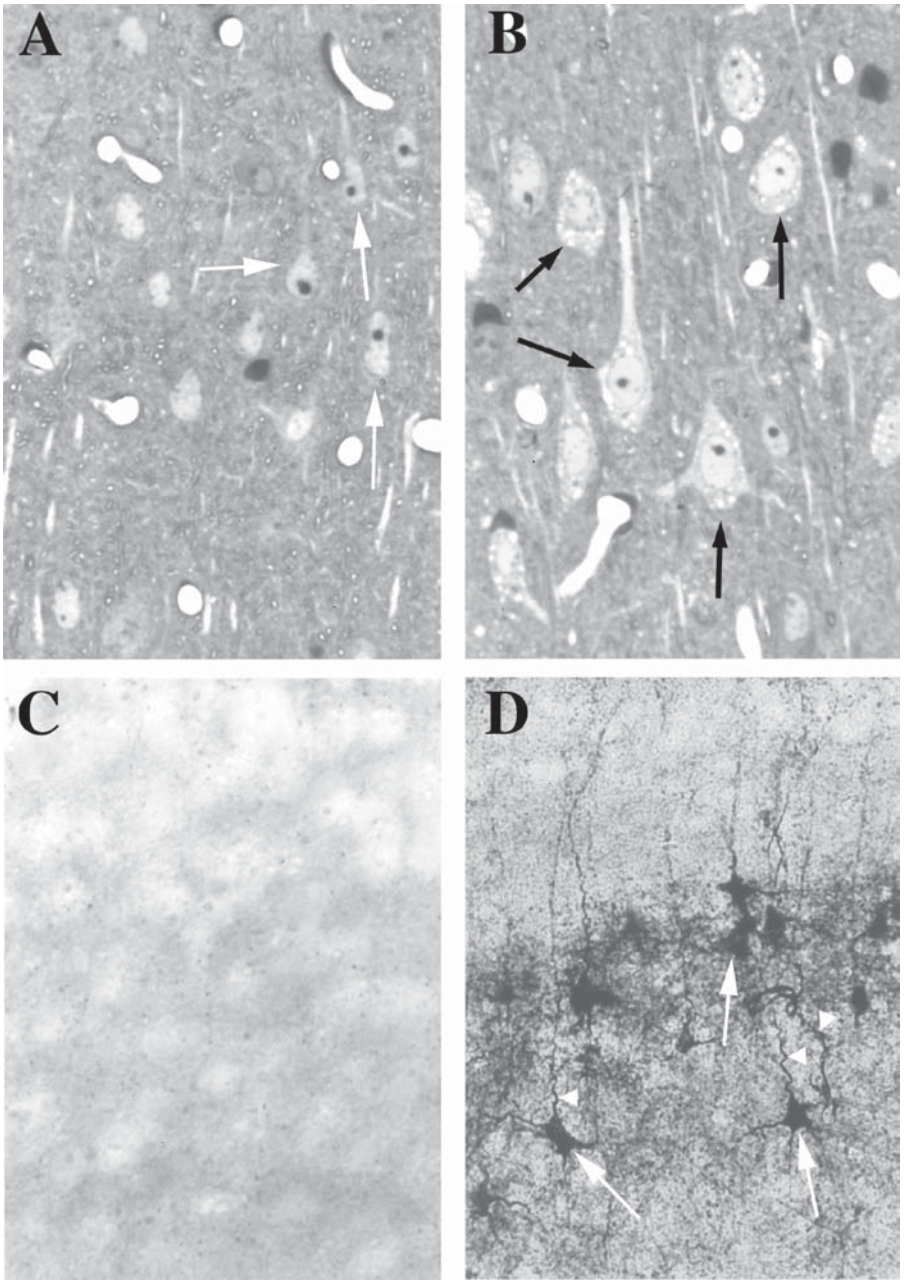

Fig. 1. NRHypo neurodegeneration. (**A**) Light photomicrograph of RSC taken from a rat treated with saline and sacrificed 4 h later. Layer IV–Va neurons (white arrows) are normal in appearance. (**B**) Light photomicrograph of RSC taken from a rat treated 4 h earlier with a low dose (0.5 mg/kg) of MK-801. Multiple layer IV–Va neurons (black arrows) are undergoing reversible NRHypo neurodegeneration. Neurons are slightly swollen and multiple vacuoles can be seen in the cytoplasm. (**C**) Light photomicrograph of RSC taken from a rat treated 4 d earlier with saline and stained with de Olmos silver technique for visualizing degenerating neurons. No neurons are agyrophilic. (**D**) Light photomicrograph of RSC taken from a rat treated with high dose of phencyclidine (PCP) (50 mg/kg), sacrificed 4 d later, and processed with de Olmos silver technique. Multiple agyrophilic neurons (white arrows) undergoing degeneration can be identified. Apical dendrites have developed a corkscrew deformity (arrowhead) and are fragmenting.

and muscarinic receptors) vulnerable corticolimbic neurons, resulting in either reversible or irreversible injury of these neurons, depending on the intensity and duration of excitotoxic stimulation *(9,10)*.

Based on several lines of evidence, it has been proposed *(11)* that the mechanism by which NMDA antagonist drugs injure cerebrocortical neurons may be relevant to the mechanism of neurodegeneration in Alzheimer disease (AD). It has been demonstrated repeatedly that the functional capacity of the NMDA receptor system becomes progressively diminished with advancing age *(12–15)*, and the diminution is greater in AD patients than healthy control subjects *(16)*.

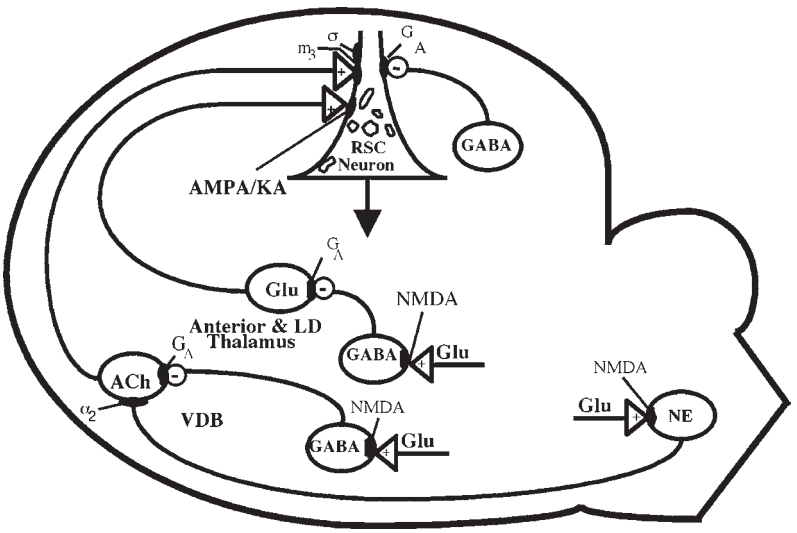

Fig. 2. Circuitry mediating NRHypo neurodegeneration. Glu acting through NMDA receptors on GABAergic and noradrenergic neurons maintains tonic inhibitory control over two major excitatory pathways that convergently innervate RSC neurons. Systemic administration of an NMDA antagonist would block NMDA receptors, thereby abolishing inhibitory control over both of the excitatory inputs to the RSC neuron. The disinhibited excitatory pathways then would simultaneously hyperactivate the RSC neuron, which would create chaotic disruption of multiple intracellular signaling systems, thereby causing immediate derangement of cognitive functions subserved by the afflicted neurons, and reversible or irreversible neuronal injury, depending on how long the disruption lasts. This circuit diagram focuses exclusively on RSC neurons. A similar disinhibition mechanism and similar but not necessarily identical neural circuits and receptor mechanisms mediate damage induced in other corticolimbic brain regions by sustained NRHypo. (+) = excitatory input; (–) = inhibitory input; ACh = acetylcholine; NE = norepinephrine; Glu = glutamate; GABA = γ-aminobutyric acid; $\alpha_2 = \alpha_2$ subtype of adrenergic receptor; $G_A = GABA_A$ subtype of GABA receptor; $m_3 = m_3$ subtype of muscarinic cholinergic receptor; AMPA/KA = AMPA/KA subtype of Glu receptor; NMDA = NMDA subtype of Glu receptor; σ = sigma site.

Thus, the NMDA receptor system in the AD brain may be considered severely hypofunctional, just as the NMDA receptor system following treatment with an NMDA antagonist drug is severely hypofunctional. It follows that if an NMDA receptor hypofunction (NRHypo) state in the adult rat brain can cause neurons in various corticolimbic brain regions to undergo excitotoxic degeneration, then an NRHypo state in the aging AD brain may also promote excitotoxic corticolimbic neurodegeneration. Interestingly, the neurons that degenerate in AD are similar in type and location to the neurons that degenerate in the adult rat brain under NRHypo conditions *(8,17)*. Moreover, it is generally agreed that loss of synaptic complexes correlates closely with cognitive deterioration in AD, and that in the NRHypo adult rat brain excitotoxic degeneration of dendritic spines and loss of synaptic complexes that are present in high concentration on these spines is a primary feature of the neuropathologic process *(8)*. In addition, there is evidence that β-amyloid deposition may accelerate the process by which the NMDA receptor system becomes hypofunctional in the aging AD brain *(18)*, so there is no conflict between β-amyloid-based and NRHypo-based hypotheses.

Data from several studies suggest that estrogen can protect against and/or retard the development of AD in susceptible individuals *(19,20)*. In addition, rodent studies have shown that estrogen can protect against neuronal death in several neurodegenerative models *(21)*. We therefore sought to determine whether estradiol, the most abundant and physiologically important estrogenic compound in the CNS, could protect against NRHypo neurodegeneration.

Results

Estradiol, when given as an acute one time dose of 100 μg, provided no protection against NRHypo neurodegeneration ($p > 0.2$; Fig. 3A). By contrast, when given chronically, it resulted in a reduction in the severity of NRHypo neurodegeneration (Fig. 3B; main effect of treatment condition: $F_{[1,43]} = 16.2$, $p = 0.0002$; treatment duration × treatment condition interaction: $F_{[1,43]} = 0.5$, $p = 0.47$). Administering 100 μg twice daily for 4.5 d produced a 27% reduction in the amount of neurodegeneration ($p = 0.01$) and 14 d produced a 35% reduction ($p = 0.009$). Although the 14-d treatment provided a numerically greater reduction in neurodegeneration compared with the 4.5-d treatment, the difference was not statistically significant (main effect of treatment duration: $F_{[1,43]} = 0.7$, $p = 0.41$). Because 100 μg of estradiol provided only moderate amounts of protection, we decided to test an even higher dose of estradiol. Although a higher daily dose of estradiol (300 μg twice daily) conferred a 25%

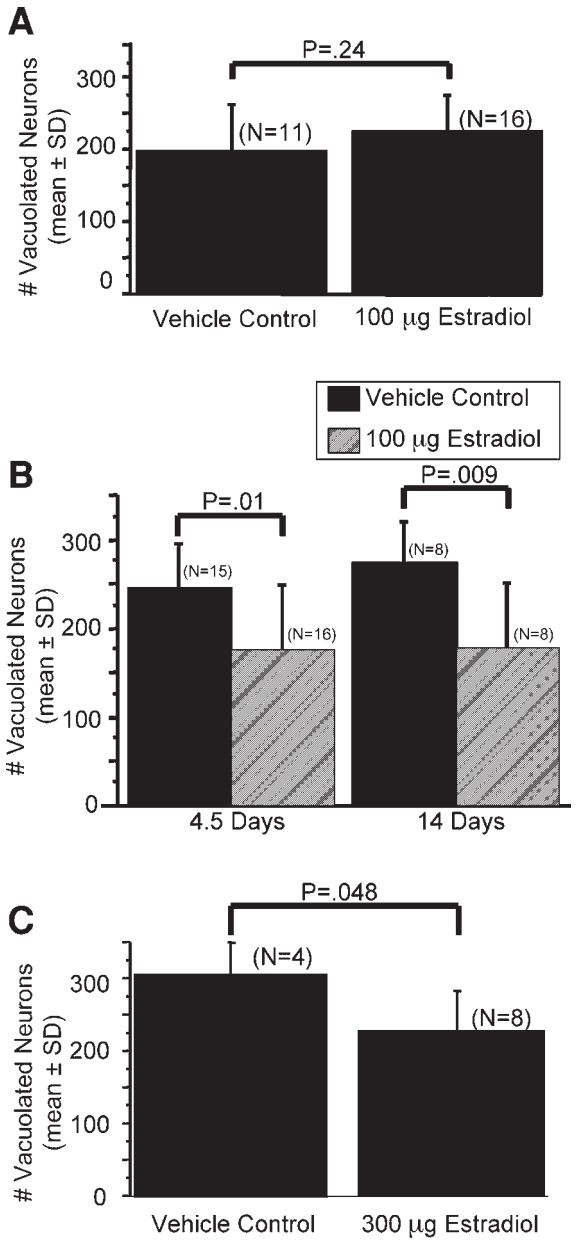

Fig. 3. Effects of chronic and acute estradiol on NRHypo neurodegeneration. (**A**) A onetime acute dose of estradiol (100 μg) provides no protection against NRHypo neurodegeneration (p = 0.24). (**B**) Chronic dosing of estradiol (100 μg twice daily) protects against NRHypo neurodegeneration (treatment duration × treatment condition: $F_{[1,43]} = 0.5$, $p = 0.47$; main effect of treatment condition: $F_{[1,43]} = 16.2$, $p = 0.0002$; main effect of treatment duration: $F_{[1,43]} = 0.7$, $p = 0.41$). The significant main effect of treatment condition was explained by the 100 μg twice daily for 4.5 d condition being 27% lower ($p = 0.01$) and the 100 ug twice daily for 14 d condition being 35% lower ($p = 0.009$) than the control conditions. (**C**) A higher dose of ED (300 μg twice daily) for 4.5 d also provided protection against NRHypo neurodegeneration ($p = 0.048$). The amount of protection (25%) was similar to that seen with 100 μg twice daily.

($p = 0.048$) protection against NRHypo neurodegeneration, the degree of protection does not appear to be any greater than that seen with 100 μg twice daily (Fig. 3C).

Discussion

The present study demonstrates that chronic estradiol treatment can protect against the neurodegenerative effects of the potent NMDA receptor antagonist MK-801. The modest degree of neuroprotection (27%) conferred by the 100-μg twice daily dosing for 4.5 d appears to be maximum in that higher doses or longer treatment did not result in significantly greater protection. Whether lower doses of estradiol are also effective is currently unknown, but given the effect produced with the 100-μg dose, it is anticipated that decreased doses would also be protective. Given the magnitude of estradiol's neuroprotective effect, a large number of animals will need to be studied at several additional doses in order to determine more precisely estradiol's dose-response curve. The effectiveness of the 100-μg dose in our study is consistent with reports that similar dosing is effective in preventing neurodegeneration secondary to hypoxia-ischemia in rodents *(21)*. Thus, we conclude that estradiol can protect against NRHypo neurodegeneration consistent with the hypothesis that the NRHypo mechanism could be a factor in the development of pathologic tissue changes in the brains of patients with AD.

The mechanism underlying estradiol's protective effect against NRHypo neurodegeneration remains to be determined. Estradiol can act through genomic mechanisms at the nuclear level *(22)*, interact with cellular receptors to activate second-messenger systems *(23)*, induce antiapoptotic gene expression *(24)*, maintain intracellular calcium homeostasis *(25)*, promote antioxidant activity *(26)*, and modulate the actions of neurotrophins *(23,27)*. Thus, the role of estradiol in the CNS is extensive and complex. Based on our understanding of the NRHypo neurodegenerative mechanism, we suspect that estradiol's protective effects could be mediated by interactions at NMDA and/or GABA receptors.

Estradiol has been shown to have a variety of actions at the NMDA receptor depending on dose and duration of treatment. Estradiol's positive influence on the glutamatergic system has been described in numerous studies based on the sensitivity of NMDA receptor–mediated synaptic input, plasticity, increased receptor-specific binding, and dendritic spine formation, with some variation depending on brain region *(28–31)*. Dosing schedules in these studies (e.g., 2 d to 2 wk) are in accordance with the neuroprotective effects seen with chronic dosing in our study. In addition to the effects seen with long-term estradiol exposure, there is evidence that estradiol can exert rapid electrophysiologic effects on NMDA receptor–mediated currents *(25,28,29, 32)*. However, the ability of estradiol to acutely alter NMDA receptor-mediated currents is an unlikely mechanism for its neuroprotection against NRHypo neurodegeneration in our model because it did not acutely affect the toxicity associated with NMDA receptor blockade. Since estradiol's protective effects are conferred with chronic dosing, its actions are most likely mediated indirectly by genomic mechanisms

such as posttranscriptional regulation of receptor density or structure, interactions with neurotrophic factors, and/or influences of second-messenger systems, rather than by direct effects at NMDA or other ionotropic receptors or direct-acting neuroprotective properties such as antioxidant effects. Consistent with our findings of a difference between acute and chronic dosing, Wong and Moss *(32)* found that 2 d of estradiol treatment produced different NMDA receptor–associated effects than did acute application of estradiol and that these dosing effects occurred via a different mechanism.

$GABA_A$ receptor agonists such as benzodiazepines and barbiturates have been shown to provide significant protection against NRHypo neurodegeneration *(7)*. Estradiol has been shown to alter GABA conduction, increase GABA release, upregulate $GABA_A$ receptors, increase $GABA_A$ receptor agonist binding affinity, and alter glutamic acid decarboxylase mRNA *(33–37)*. In our model of disinhibition, one of the key components underlying NRHypo neurodegeneration involves the disinhibition of a complex multisynaptic circuit (Fig. 2). A key element of the circuit is that GABAergic interneurons have NMDA receptors on their surface and receive constant stimulatory input via these receptors causing a tonic inhibitory output. In the NRHypo condition, these GABAergic neurons are no longer stimulated adequately, and they lose their ability to inhibit the two key excitatory pathways (cholinergic output from the basal forebrain and glutamatergic output from the thalamus), resulting in exitotoxic damage. In a recent study in which Rudick and Woolley *(37)* examined the effects of estradiol on inhibitory synaptic input to hippocampal cells, they demonstrated that after an early transient suppression of $GABA_A$-mediated inhibition, there is a concurrent enhancement of both $GABA_A$ and NMDA synaptic input to hippocampal CA1 pyramidal cells after 48 h of estradiol treatment. Either of these effects could account for the observed neuroprotective effects with chronic estradiol treatment in our study. Although estradiol's influences at NMDA receptors and $GABA_A$ receptors provide a plausible explanation of its protective effects associated with NRHypo neurodegeneration, its actions in the CNS are complex. An increased understanding of estradiol's mechanism in preventing NRHypo neurodegeneration could provide further insight into its protective mechanism in neurodegenerative disease processes, such as AD, in which an NRHypo state might be operative.

Materials and Methods

Adult female Sprague-Dawley retired breeders (Harlan, Indianapolis, IN) were used in all experiments. Estradiol benzoate (Sigma, St. Louis, MO) in sesame oil injected subcutaneously was used for all experimental groups, and sesame oil alone injected subcutaneously was used for control groups. Three experimental conditions were studied: (1) acute study: 100 μg of estradiol × 1 dose ($n = 16$); (2) chronic study: 100 μg of estradiol twice daily for 4.5 d ($n = 16$) or 14 d ($n = 8$); (3) high-dose study: 300 μg of estradiol twice daily for 4.5 d ($n = 8$). MK-801 (0.5 mg/kg subcutaneously; Sigma) was given 2 h after the last dose of estradiol (or control vehicle) in all experiments. Four hours after the administration of MK-801, the animals were deeply anesthetized with chloral hydrate and perfused with fixative (1% paraformaldehyde, 1.5% glutaraldehyde in phosphate buffer at pH 7.4) through the ascending aorta. The brains were removed and cut into 1-mm-thick coronal slabs, postfixed in 1% osmium tetroxide, dehydrated in a series of alcohols, cleared in toluene, and embedded flat in araldite. Histologic sections were cut 1-μm thick with an MT 2C Sorvall Ultratome using 0.5-in.-wide glass knives. The sections were then stained with a mixture of methylene blue and azure II for light microscopy. Vacuolated RSC neurons (Fig. 1B) were counted on each side of the brain at a level where the neurotoxic reaction is known to be near maximal (approx 5.5 mm caudal to bregma, a level that is easily identified because it is where the corpus callosum ceases decussating across the midline). The investigator quantifying the reaction was blind to the treatment conditions.

Statistical significance was determined by an unpaired *t*-test in the acute and high-dose studies. In the chronic treatment study, an analysis of variance model with two between-subject independent variables (treatment condition and duration) and one dependent measure (severity of neurotoxicity) was used to analyze the results.

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