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13. ABSTRACT *(Maximum 200 Words)*
In year 1 of this grant, we have made good progress toward meeting our stated objectives. For Objective 1, we have; A. established that administration of kainic acids to rats at a dose of 8.5 mg/kg results in consistent and reliable siezure behavior and that survival times of 96 hr post-injection is optimal for detection of brain injury and B. established that progesterone can protect animals from seizures produced by kainate, and that a relatively low dose of progesterone is effective in conveying this effect. In addition, we are beginning studies to examine the effects of co-administration of estrogen on the neuroprotective effects of progesterone. For Objectives 2 and 3, we have; established that transient (30 min) exposure to glutamate (100 μM) produced reliable and consistent neuronal death after 24 hr. Furthermore, our studies have established that addition of progesterone does not protect rat cerebellar neurons against glutamate-induced excitotoxic neuronal death. We are extending these studies to cultures of cortical neurons that may possess more progesterone receptors, and are beginning to examine the effects of estrogen and co-administration of estrogen and progesterone on glutamate-induced excitotoxicity.

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Table of Contents

Introduction	4
Body	4
Key Research Accomplishments	26
Reportable Outcomes	26
Conclusions	26
References	27

(4) INTRODUCTION:

Progesterone and Neuroprotection

Excitotoxicity contributes significantly to neuronal death and neurological impairment after both acute and chronic injuries; e.g., ischemia, head trauma, neurotoxicity, and stress related disorders. It is becoming increasingly clear that reproductive hormonal status can modulate brain excitability; generally, estrogen increases and progesterone decreases excitability to a number of stimuli, both natural and artificial. As women become more active in the military, issues of the interaction of cyclic changes in gonadal steroids on injury incurred during military service become relevant. Recent evidence indicates that the gonadal steroid progesterone greatly attenuates the excitotoxicity that leads to neuronal cell loss. The mechanisms whereby progesterone exerts such protective actions are not defined. The **working hypothesis** for the proposed studies is that progesterone treatment can protect or intervene against military-related brain injury due to toxicity, head trauma, and ischemia and that one mechanism of neuroprotection involves upregulation of mitochondrial gene expression.

(5) BODY:

The **specific hypotheses** to be tested are as follows:

1. **Progesterone protects against neuronal death caused by kainic acid-induced status epilepticus *in vivo* and excitotoxicity-induced neuronal death** *in vitro.*
2. **Excitotoxic levels of glutamate down-regulate the expression of mitochondrial genes, e.g., cytochrome oxidase, both *in vitro* and *in vivo.***
3. **A mechanism of neuroprotection by progesterone is the up-regulation of mitochondrial gene expression, thereby increasing the resistance of neurons to excitotoxicity.**

APPROVED STATEMENT OF WORK

Year 1

Objective 1

- Order necessary supplies for progesterone capsule implantation.
- Establish effective dose and time course for progesterone's neuroprotective actions.
- Begin to examine the effect of estrogen co-administration.

Objective 2 and 3

- Order necessary supplies for measurements of mitochondrial gene expression, cell culture, cell death assays, and mitochondrial functional assays.
- Begin to establish dose-response relationships for glutamate and cell death using primary neuronal cultures.
- Begin measurements of COX III RNA, COX protein, and COX enzyme activity on histological samples generated by experiments described in Objective 1.

Objective 1: Progesterone and Neuroprotection

What are the relative protective effects of progesterone against neuronal death?

The first series of experiments were conducted to: (1) establish an effective dose of kainic acid that produced reliable and repeatable epileptic seizures (status epilepticus; SE) with low mortality; (2) determine the optimal survival period post-kainate administration that resulted in consistent cell death in limbic areas (i.e. entorhinal cortex, hippocampus); and (3) test whether high plasma progesterone levels were neuroprotective against cell death produced by kainic acid-induced status epilepticus (KASE).

All experiments were conducted using ovariectomized (ovx'd) female rats (Sprague-Dawley, Zivic Miller). At the time of ovariectomy, animals were implanted subcutaneously with 6 silastic capsules (3 cm) containing either progesterone crystals (Sigma) or saline to produce plasma progesterone levels of approximately 60-80 ng/ml or <5 ng/ml respectively. This dose was chosen based on our previous studies showing that lactating animals (PROG levels 60-80 ng/ml) were insensitive to the NMDA agonist NMA (Ren et al., 2000). Seven days after capsule implantation, animals were injected with kainic acid (5, 7, 8, 8.5, 9, 10 mg/kg, i.p.; Sigma). Behavioral observations were conducted from 0-6 hr. post-injection, and seizure behaviors scored using the Lothman and Collins Seizure Activity Scale (LCSAS). Animals were sacrificed at 12, 48, and 96 hr post-kainate by perfusion and their brains removed and stored in 20% sucrose solution. Plasma samples were obtained at the time of euthanasia for subsequent determination of progesterone levels using radioimmunoassay (Diagnostic Products, Coat-A-Count). To visualize neuron patterns, brains were sectioned at 35 μm and stained using an antibody directed against the neuronal marker neuron-specific nuclear antigen (Chemicon). Limbic regions were examined for cell death by an investigator blind to condition.

The results of these studies established that kainic acid administration at a dose of 8.5 mg/kg produced reliable and consistent seizures with little mortality (<10%). Administration of kainic acid at a dose of 8 mg/kg or less produced inconsistent seizure activity (i.e. animals rarely scored higher than stage 1 or 2 on the LCSAS); by contrast, doses exceeding 9 mg/kg resulted in high mortality rates (>80% of the animals reached stage 5).

Brain morphological patterns were examined at 12, 48 and 96 hr post-kainate administration (8.5 mg/kg). At 12 hr post-KA, very little neuronal damage was detected; at 48 hr, some cell death was apparent within the entorhinal cortex and the CA1 region of the

hippocampus. By 96 hrs post-KA, neuronal loss was consistently observed across animals within several limbic regions.

These studies established that administration of kainic acid at a dose of 8.5 mg/kg resulted in consistent and reliable seizure behavior and that survival times of 96 hrs post-injection was optimal for detection of brain injury.

Using the 8.5 mg/kg dose, we next examined whether progesterone administration was effective in attenuating seizure activity and the consequent brain damage. As shown in Figure 1, animals with progesterone levels of +60 ng/ml displayed reduced seizure severity scores (Figure 1) and a reduction in the number of stage 4 behaviors based on the LCSAS (Figure 2). Accompanying the reduction in seizure severity was a striking reduction in neuron loss in limbic regions (Figure 3).

NOTE: During completion of these studies, a world-wide shortage in the seaweed from which kainic acid is extracted occurred resulting in an international shutdown in production (i.e. no kainate was commercially available). We substituted the convulsant pilocarpine, a muscarinic cholinergic agonist as a seizurogenic agent. Doses of pilocarpine ranging from 100-400 mg/kg were tested and found to produce seizure activity that was very distinct from KASE in that it was very rapid in onset, severe in nature, and was not associated with neuronal loss in limbic regions. Fortunately, while collecting dose-response data for pilocarpine, production of kainic acid resumed and became commercially available.

What is the effective dose of Progesterone necessary to convey neuroprotection?

All experiments were conducted using ovx'd female rats (Sprague-Dawley, Zivic Miller). At the time of ovariectomy, animals were implanted subcutaneously with 2, 4 or 6 silastic capsules (3 cm) containing either progesterone crystals or saline. This produced plasma progesterone levels of 20, 40 or 60 ng/ml respectively. Animals receiving blank capsules had progesterone plasma levels of <5 ng/ml. Seven days after capsule implantation, animals were injected with kainic acid (8.5 mg/kg, i.p.; Sigma). Behavioral observations were conducted from 0-6 hr. post-injection, and seizure behaviors scored using the Lothman and Collins Seizure Activity Scale (LCSAS). Animals were sacrificed at 96 hr post-kainate by perfusion and their brains removed and stored in 20% sucrose solution. Plasma samples were obtained at the time of euthanasia for subsequent determination of progesterone levels using radioimmunoassay. To visualize neuron patterns, brains were sectioned at 35 μm and stained using an antibody directed against the neuronal marker neuron-specific nuclear antigen (Chemicon). Limbic regions were examined for cell death by an investigator blind to condition.

Treatment of animals with progesterone resulted in an overall reduction in seizure severity. Dose response data indicated that plasma progesterone levels of approximately 20 ng/ml resulted in a significant reduction in both seizure severity scores (Figure 4) and number of stage 4 behaviors (Figure 5). A detail analysis of the stage 4 behaviors is shown in Figure 6. We are currently examining the brains from these animals for morphology and cell death.

Together, these studies established that progesterone can protect animals from seizures produced by kainate, and that a relatively lower dose of progesterone is effective in

conveying this effect. We are currently extending these studies to examine the potential neuroprotective effects of even lower doses of progesterone. In addition, we are beginning studies to examine the effects of co-administration of estrogen on the neuroprotective effects of progesterone.

Objective 2 and 3: Progesterone and protection against excitotoxic neuronal death *in vitro*.

The first series of experiments were done to test the protective effect of progesterone against glutamate-induced neuronal death in cellular models of excitotoxicity. We propose to use rat cerebellar granule neurons and rat cortical neurons as models of excitotoxicity. Our initial experiments were done on rat cerebellar granule neurons and we propose to extend these studies to rat cortical neurons.

MATERIALS AND METHODS

Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national and international laws and policies (NIH guide for the Care and Use of Laboratory Animals, NIH publication no. 85-23,1985).

Primary rat cerebellar cultures:

Cerebellar granule cell cultures were prepared from 7-day old Sprague-Dawley rats using standard method [3]. Neurons were seeded at a density of 2 x10^6 cells/ml into poly-L-lysine (MW 30 000 - 700 000)-coated 6-well tissue culture chambers and cultured in Eagle's Basal Medium supplemented with Earle's salts, 10% inactivated fetal calf serum, 25 mM KCl and gentamycin (50 ng/ml). To prevent growth of glial cells, cytosine arabinoside (10 μM) was added to the

cultures 24h after seeding. In order to distinguish the effect of progesterone from the effects of other hormones in the serum, the medium was replaced after 4 days in culture (4 DIV) with fresh medium containing hormone-free serum. The neuronal cultures were maintained in hormone-free medium for another 4 days. The neuronal cultures maintained in hormone-free medium do show sensitivity to glutamate (Figure. 1).

Excitotoxicity:

Cultures were exposed to glutamate (50 - 250 μM) in a Locke solution (134-mM NaCl, 25 mM KCl, 4 mM $NaHCO_3$, 5 mM HEPES, 2.3 mM $CaCl_2$ and 5 mM glucose) for 30 min in the presence of 10 μM glycine. After exposure to glutamate, the cells were washed and kept in the old culture medium without glutamate for up to 24 hr. Control cultures were treated with the vehicle for the same time period as that of glutamate treated cells.

The effect of progesterone on glutamate-induced excitotoxicity was determined using the following conditions:

Pretreatment: Progesterone (10 -1000 nM) was added to the culture medium 2 days prior to exposure to glutamate. The cultures were exposure to glutamate in Locke solution in absence of added progesterone.

Pre- and Post treatment: Progesterone (10 - 1000 nM) was added to the culture medium 2 days prior to exposure to glutamate, progesterone was added to Locke solution 30 min before the addition of glutamate and progesterone was also to the culture medium after glutamate exposure.

Post treatment: Progesterone (10 – 1000 nM) was to the culture medium only after exposure to glutamate.

Cell viability:

Cell viability was determined using a two-color fluorescence assay based on the simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cell viability – intracellular esterase activity and plasma membrane integrity. Viable granule cell neurons were quantified after staining of cells with cell-permeant calcein AM (2 μM). Nonviable granule cell neurons were quantified with cell-impermeant propidium iodide (10 μg/ml). The number of cells that displayed PI fluorescence (nonviable) to the total number of cells in a field was determined.

Drugs:

In experiments with cell cultures, progesterone was dissolved in 60% alcohol and the final alcohol concentration was less than 0.01%. Cultures were treated with the vehicle alone were used as controls. Stock solution of NMDA-antagonist, MK-801, and glycine antagonist, 7-chlorokynurenic acid (7-CKYN), were prepared in water and added prior to the addition of glutamate.

Statistical analysis

Results are expressed as the mean ± S.E.M. Differences between controls and test samples were evaluated by ANOVA: Fischer's F-test was first used to compare between-groups and within-groups variance; if the former was significantly ($P<0.05$) higher than the latter, individual groups were compared by Tukey's test for multiple comparisons [1].

RESULTS

Effect of glutamate on cell death in cerebellar granule neurons:

We initially examined the effects of glutamate exposure on granule cell viability over a broad range of concentrations. Propidium iodide (PI) dye was used to monitor the dead cells. Calcein AM was used to monitor viable cells. Propidium iodide is a membrane-impermeant chromatin dye that is excluded from cells with intact membranes, whereas neurons with damaged cell membranes emit red fluorescence as the dye intercalates with DNA. Calcein AM is a nonfluorescent membrane-permeant dye when deesterified (only by living cells), emits a green fluorescence. Data from a representative experiment are shown in Figures 7 and 8. The neurotoxicity produced by a 30min exposure to glutamate in Locke solution increased with increasing concentrations of glutamate (Fig. 8; 60 to ± 7% with 100 μM glutamate and 90 ± 5% with 250 μM glutamate; measured on sister cultures in 5 different preparations). No difference in glutamate-induced neurotoxicity was observed between cerebellar granule neurons maintained in normal serum vs. hormone-free serum. The glutamate-induced neurotoxicity was blocked by the selective NMDA-receptor antagonist MK-801 (10 μM, Fig.8) and by the glycine antagonist 7-chlorokynurenic acid (10 μM, Fig. 8).

The results of these studies established that transient (30 min) exposure to glutamate (100 μM) produced reliable and consistent neuronal death after 24h.

Lack of protection by progesterone against glutamate-induced excitotoxic cell death in rat cerebellar granule neuronal cultures:

Using the protocol of 30min exposure of 100 μM glutamate, we evaluated the protective effect of addition of progesterone against neuronal death. Since the serum used in the culture medium contains hormones including progesterone, we evaluated the effect of substitution of serum with hormone-free serum on the viability of the neuronal cultures. Cerebellar granule neurons were plated in presence of serum for the first 4 days and the culture medium was replaced with fresh medium containing hormone-free serum. Under these conditions, the neuronal cultures appear to retain similar morphological features as that of cultures maintained in serum containing medium (Compare Figs. 7A and 9A). Addition of glutamate induced similar neurotoxicity (Compare Figs. 8 and 10). Addition of progesterone (10 – 1000 nM) either before (pre in Fig. 10) or during (pre and post in Fig. 10) the exposure to glutamate failed to provide significant protection against neuronal death (Figures. 9 and 10). In order to evaluate the significance of lack of protection by progesterone against excitotoxic cell death in rat cerebellar granule neurons, we have begun similar experiments with rat cortical neurons. The rationale for choosing cortical neuronal cultures is that progesterone receptors are highest in frontal cortex and lowest in cerebellum [2].

Together, these studies established that addition of progesterone do not protect rat cerebellar neurons against glutamate-induced excitotoxic neuronal death. We are currently extending these studies to cortical neuronal cultures to examine the potential neuroprotective effects of progesterone against excitotoxicity *in vitro*. In addition, we are beginning studies to examine the effects of estrogen and co-administration of estrogen and progesterone on glutamate-induced excitotoxicity.

FIGURE LEGENDS

Figure 1. Reduction of severity of kainate-induced seizures by progesterone. Mean number of Stage 4 behaviors displayed following saline or kainic acid (KA; 9 mg/kg; i.p.). Two weeks prior to KA administration, animals were ovariectomized and implanted with capsules containing either progesterone (PROG) or cholesterol (blank).

Figure 2. Reduction in incidence of Stage 4 behaviors by progesterone following kainate-induced seizures. Composite seizure severity scores following saline or kainic acid (KA) administration. Two weeks prior to KA administration, animals were ovariectomized and implanted with six capsules containing either progesterone (PROG; P6) or cholesterol (blank).

Figure 3. Reduction of hippocampal neuronal death by progesterone following kainate treatment. Photomicrographs of Nissl stained hippocampus sections following kainic acid (KA) administration in ovariectomized (OVX+KA; top panel) and ovariectomized and progesterone replaced (OVX-P+KA; middle panel) female rats. Lower panels show higher magnifications of the boxed region depicted. Note the profound neuronal loss within the CA1 region following KASE in non-progesterone treated animals.

Figure 4. Progesterone dose-dependence of reduction in severity of kainate-induced seizures by progesterone. Composite seizure severity scores following kainic acid status epilepticus. Two weeks prior to kainic acid (KA) administration, animals were ovariectomized and implanted with capsules containing either progesterone (PROG) or cholesterol (blank). Dose of progesterone was adjusted by varying the number of implants: P2, 2 implants; P4, 4 implants; P6, 6 implants.

Figure 5. Progesterone dose-dependence of reduction in incidence of abnormal behavior following kainate treatment. Percentage of animals displaying the most serious Stage 4 behaviors following kainic acid status epilepticus. Progesterone levels were adjusted by varying

the number of capsules implanted: P2, 2 implants; P4, 4 implants; P6, 6 implants. Control animals (Blank+Saline) did not display any stage four behaviors (data not shown).

Figure 6. Progesterone dose-dependence of reduction in the incidence of various stage 4 behaviors following kainate treatment. Detailed analysis showing the percentage of animals displaying all Stage 4 behaviors following kainic acid status epilepticus. Progesterone levels were adjusted by varying the number of capsules implanted: P2, 2 implants; P4, 4 implants; P6, 6 implants. Control animals (Blank+Saline) did not display any stage four behaviors (data not shown).

Figure 7. Glutamate-induced excitotoxic neuronal death in rat cerebellar neuronal culture. At 8 DIV, the cell culture medium was removed and exposed to glutamate (100 μM) in Locke solution for 30 min. The neurons were then cultured in normal medium without glutamate for the next 24h. Photographs were made under bright field and propidium iodide fluorescence microscopy under dark field. Representative fields from a single experiment is shown in the figure. (A) Untreated culture - bright field. (B) Culture treated with glutamate for 30 min [glutamate (100 μM) plus glycine (10 μM)] - bright field. (C) Culture treated with glutamate for 30 min followed by 24h incubation in culture medium - bright field. (D) Untreated culture - dark field. (E) Culture treated with glutamate - dark field. (F) Culture treated with glutamate for 30 min followed by 24h incubation in culture medium - dark field. Note at 24 h following a 30 min exposure to glutamate, there is a significant increase in the number of PI positive cells.

Figure 8. Dose-response relationship for glutamate-induced neurotoxicity. Viable neurons are stained green with calcein AM whereas nonviable neurons stain positive with propidium iodide. At each tested concentration, the mean and SEM of rat cerebellar neurons that stained positive with propidium iodide are expressed as a percentage of the total cell count. Sister cultures were

used for each concentration and the experiments were repeated in 5 different culture preparations. MK-801 (10 μM) and 7-CKYN (10 μM) were added 10 min before the addition of glutamate.

Figure 9. Effect of addition of progesterone on glutamate-induced neuronal death. Progesterone (100 nM) was added to the hormone-free culture medium at 4 DIV as described in Materials and Methods. At 8 DIV, the neuronal culture was replaced with Locke solution containing progesterone. Glutamate (100 μM) and glycine (10 μM) were added to the culture medium for 30 min. The neurons were then cultured in normal medium without glutamate but with added progesterone for the next 24h. Photographs were made under bright field and propidium iodide fluorescence microscopy under dark field. Representative fields from a single experiment is shown in the figure but the experiments were repeated at least three times with identical results. (A) Culture treated with glutamate for 30 min in presence of progesterone [glutamate (100 μM) plus glycine (10 μM) plus progesterone (100 nM)] - bright field. (B) Culture treated with glutamate in the presence of progesterone for 30 min followed by 24h incubation in culture medium - bright field. (C) Cultures treated with progesterone alone – bright field (D) Culture treated with glutamate in the presence of progesterone for 30 min - dark field. (E) Culture treated with glutamate in the presence of progesterone for 30 min followed by 24h incubation in culture medium - dark field and (F) Cultures treated with progesterone alone – dark field.

Note that the addition of progesterone did not protect rat cerebellar cultures against glutamate-induced excitotoxic neuronal death.

Figure 10. Dose-response relationship for glutamate-induced neurotoxicity in presence of added progesterone (100 nM). Viable neurons are stain green with calcein AM whereas nonviable neurons stain positive with propidium iodide. At each tested concentration, the mean and SEM

of rat cerebellar neurons that stained positive with propidium iodide are expressed as a percentage of the total cell count. Sister cultures were used for each concentration and the experiments were repeated in 5 different culture preparations.

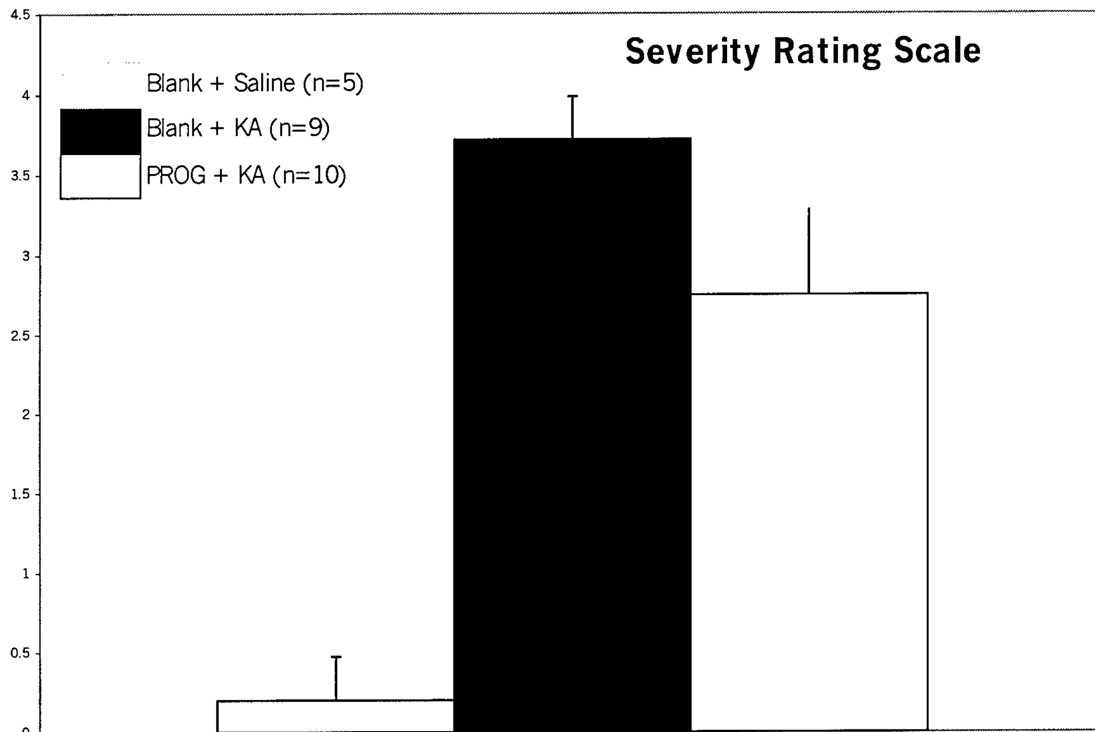


Figure 1. Reduction of severity of kainate-induced seizures by progesterone.

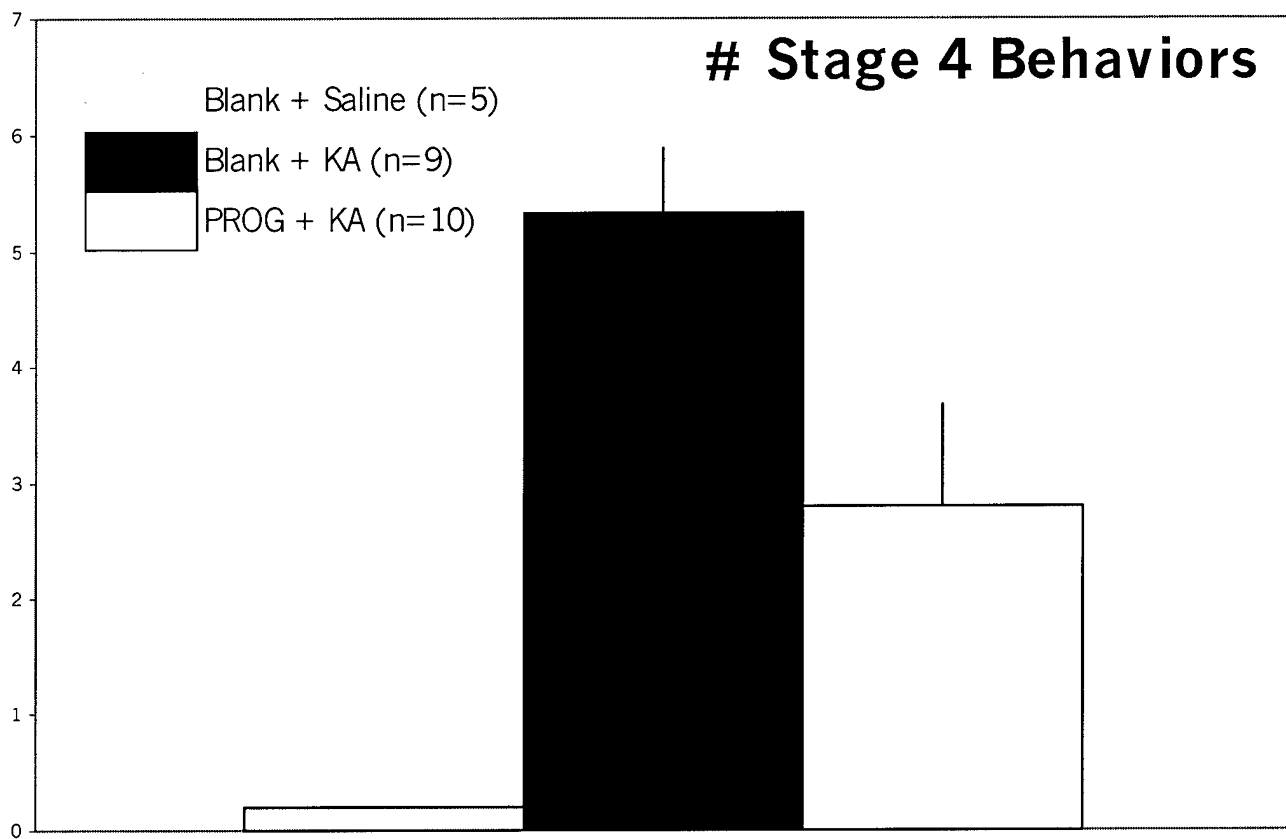


Figure 2. Reduction in incidence of Stage 4 behaviors by progesterone following kainate-induced seizures.

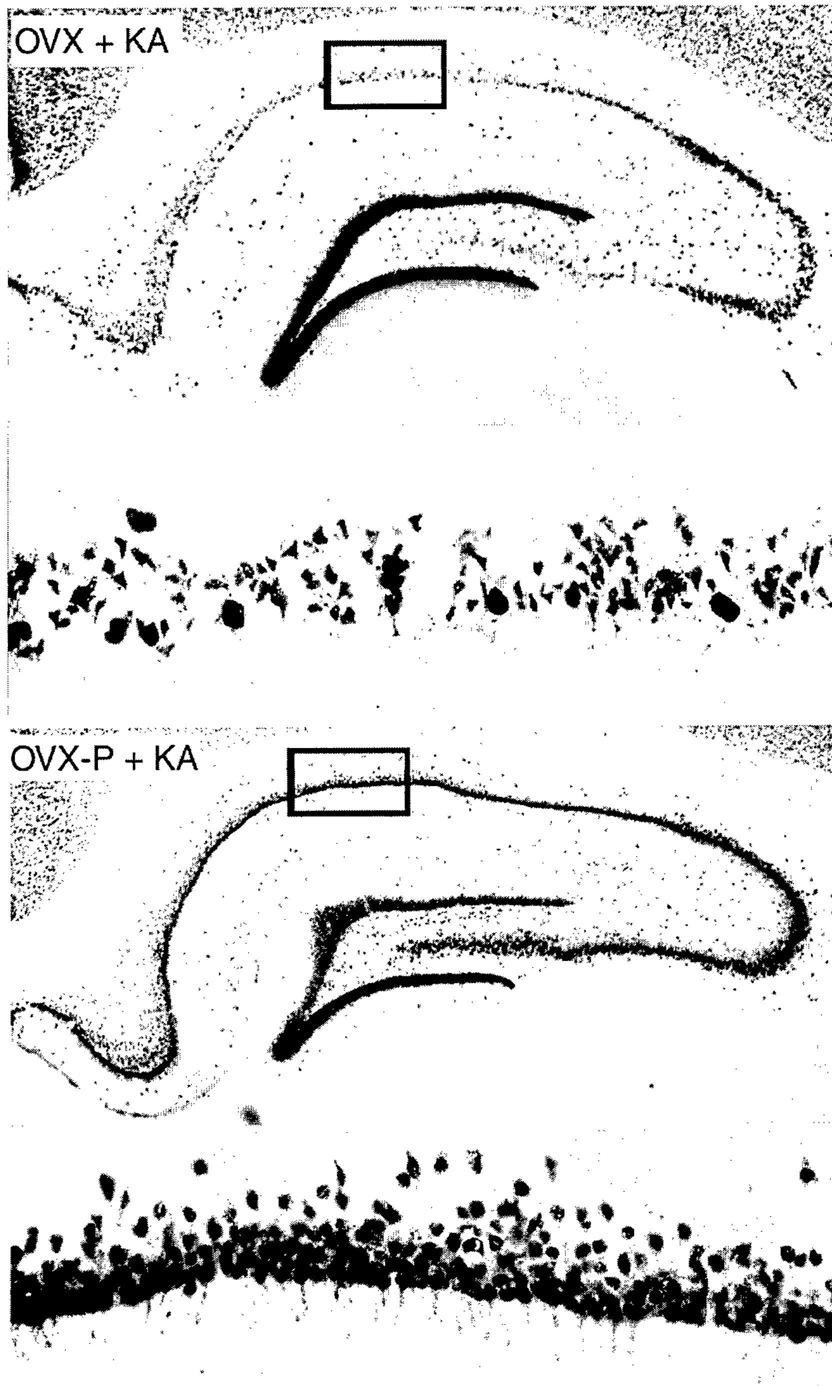


Figure 3. Reduction of hippocampal neuronal death by progesterone following kainate treatment.

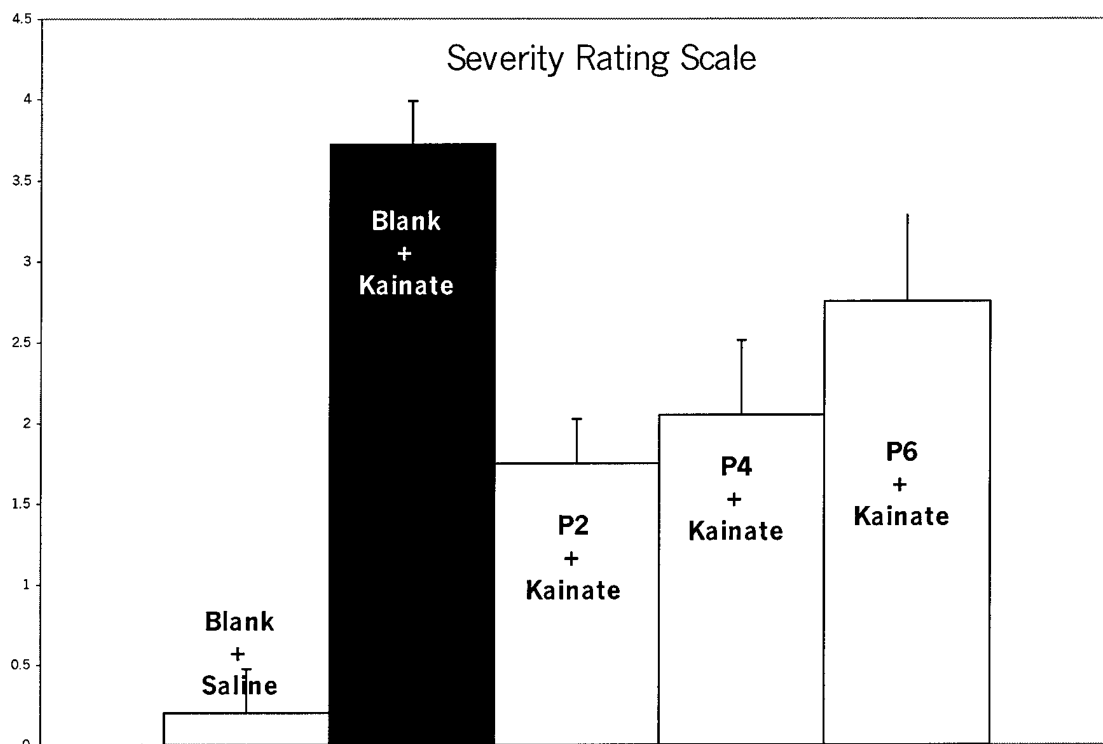


Figure 4. Progesterone dose-dependence of reduction in severity of kainate-induced seizures by progesterone.

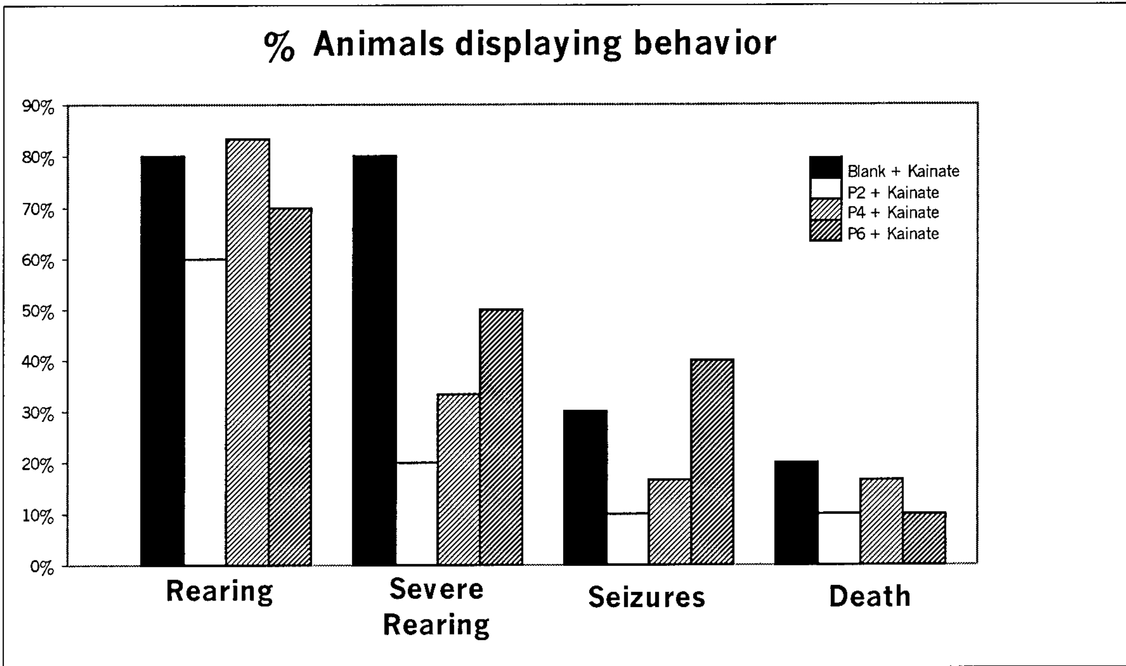


Figure 5. Progesterone dose-dependence of reduction in incidence of abnormal behavior following kainate treatment.

% animals displaying various stage 4 behaviors

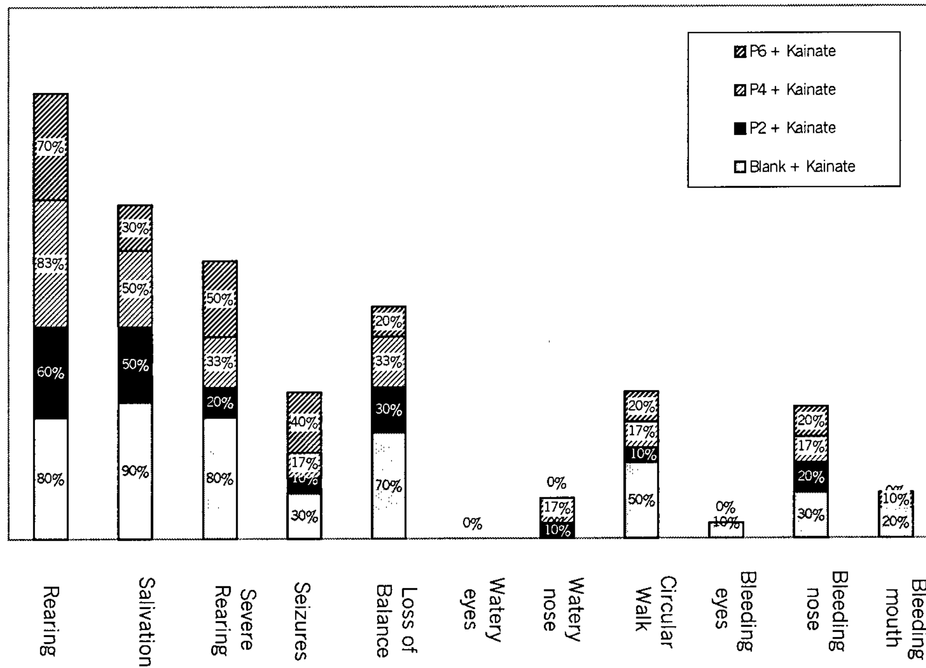


Figure 6. Progesterone dose-dependence of reduction in the Incidence of various stage 4 behaviors following kainate treatment.

Glutamate-induced Excitotoxic Cell death in Rat Cerebellar Primary Neuronal Cultures

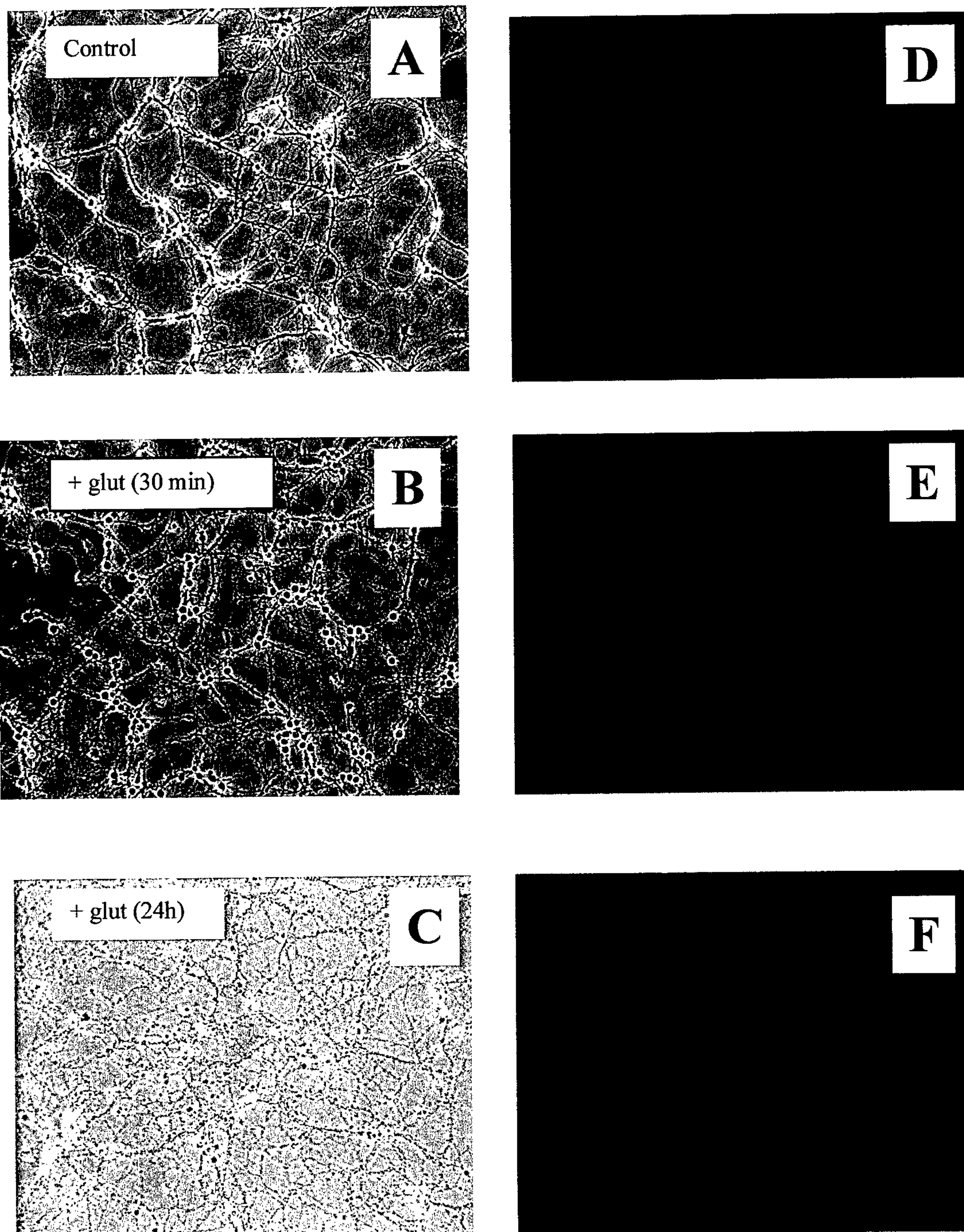


Figure 7. Glutamate-induced excitotoxic neuronal death in rat cerebellar neuronal culture

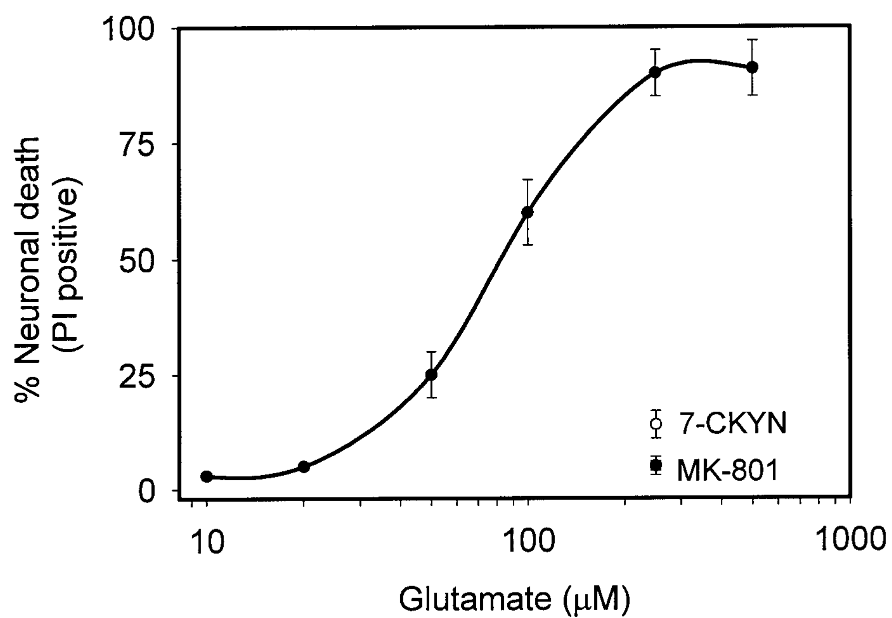
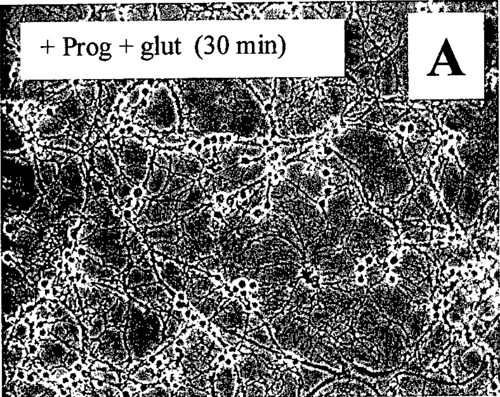
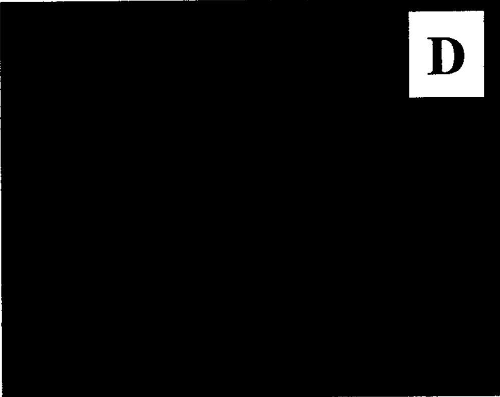
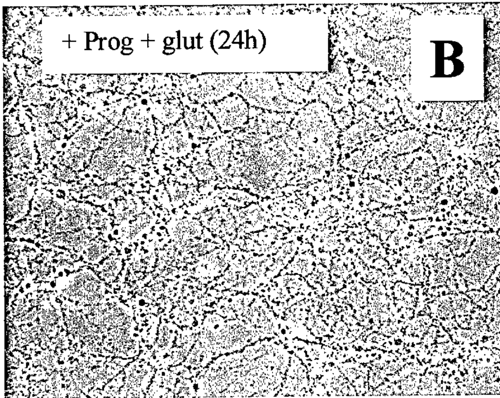


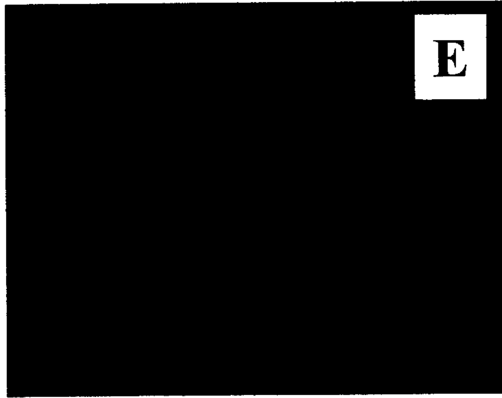
Figure 8. Dose-response relationship for glutamate-induced neurotoxicity

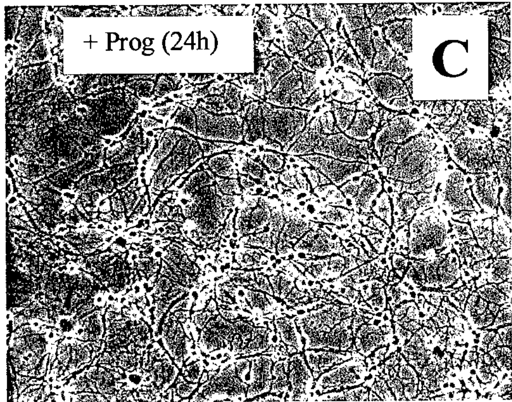
Progesterone Effect on Glutamate-induced Excitotoxicity in Cerebellar Granule Neurons











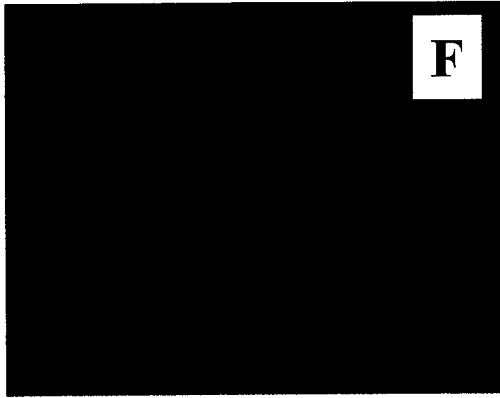


Figure 9. Effect of addition of progesterone on glutamate-induced neuronal death

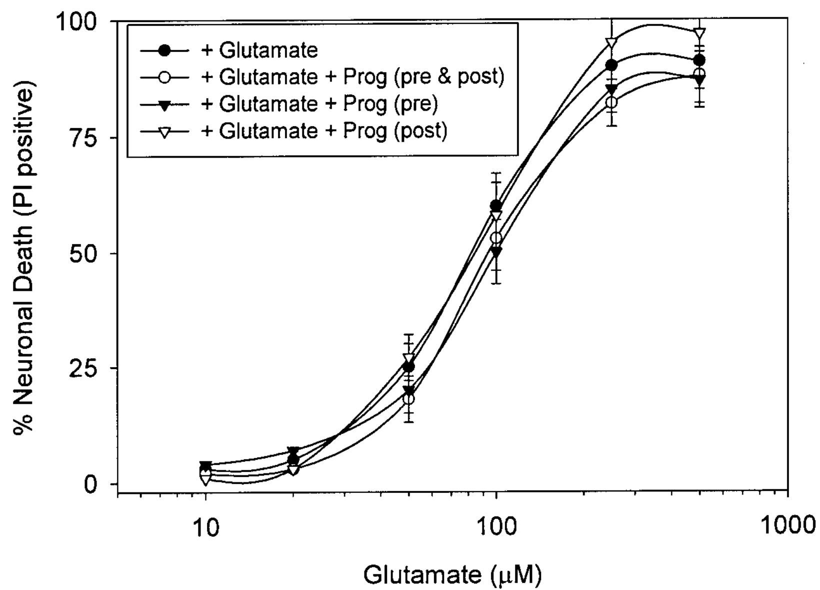


Figure 10. Dose-response relationship for glutamate-induced neurotoxicity in presence of added progesterone (100 nM)

(6) Key Research Accomplishments

- Established dose-dependent protection against kainate-induced seizure activity and incidence by progesterone.
- Confirmed that progesterone does not protect against excitotoxic cell death using cultures of cells (cerebellar granule cells) obtained from a location within the brain known to possess relatively few progesterone receptors.

(7) Reportable Outcomes **None**

(8) Conclusions

Objective 1:

Established that administration of kainic acids to rats at a dose of 8.5 mg/kg results in consistent and reliable siezure behavior and that survival times of 96 hr post-injection is optimal for detection of brain injury.

Established that progesterone can protect animals from seizures produced by kainate, and that a relatively low dose of progesterone is effective in conveying this effect.

In addition, we are beginning studies to examine the effects of co-administration of estrogen on the neuroprotective effects of progesterone.

Objectives 2 and 3:

Established that transient (30 min) exposure to glutamate (100 μM) produced reliable and consistent neuronal death after 24 hr. Furthermore, our studies have established that addition of progesterone does not protect rat cerebellar neurons against glutamate-induced excitotoxic neuronal death. We are extending these studies to cultures of cortical neurons that may possess more progesterone receptors, and are beginning to examine the effects of estrogen and co-administration of estrogen and progesterone on glutamate-induced excitotoxicity.

(9) References

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(10) Appendicies: None