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Mechanism of progesterone neuroprotection of rat cerebellar Purkinje cells following oxygen–glucose deprivation

A. Ardeshiri, **M. H. Kelley**, **I. P. Korner**, **P. D. Hurn**, and **P. S. Herson**
Department of Anesthesioloy and Peri-Operative Medicine, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97201, USA

Abstract

The survival of rat Purkinje cell (PCs) cerebellar cultures was used to test the hypothesis that progesterone is protective againstoxygen–glucose deprivation through potentiation of $GABA_A$ receptor activity. Electrophysiological recordings confirm that PCs develop robust excitatory and inhibitory synapses in culture. Exposure of cultured PCs to increasing concentrations of progesterone during oxygen–glucose deprivation revealed a concentration-dependent protection by progesterone, with significant protection observed at physiological concentrations, as low as 10 nm. The concurrent application of the $GABA_A$ receptor antagonist picrotoxin (100 μm) completely abolished the neuroprotection afforded by progesterone, indicating that progesterone is neuroprotective through activation of $GABA_A$ receptors. Progesterone potentiates $GABA_A$ receptor activity indirectly through its metabolites, such as allopregnanolone (ALLO). Therefore, ALLO was applied to PC cultures and was observed to produce significant protection at all concentrations tested, from 10 to 1000 nm. Finally, the inhibition of progesterone metabolism with finasteride abolished the protection afforded by progesterone without having any effect on the neuroprotection caused by ALLO. These data indicate that progesterone protects cerebellar PCs at physiological concentrations through a GABA-active metabolite.

Keywords

allopregnanolone; excitotoxicity; $GABA_A$ receptor; ischemia

Introduction

It is now well known that the progesterone family of steroids reduce brain injury after focal and global cerebral ischemia and after traumatic brain injury (TBI; for review see Stein, 2005). Progesterone has been reported to be effective in models of middle cerebral artery occlusion in rodents of both sexes (Jiang *et al*., 1996; Murphy *et al*., 2002; Gibson & Murphy, 2004), and in global ischemia models in rodents and cats (Gonzalez-Vidal *et al*., 1998; Cervantes *et al*., 2002; Morali *et al*., 2005). The steroid interacts with a variety of beneficial cellular and molecular signalling pathways in brain. For example, progesterone acts as an antioxidant to reduce lipid peroxidation (Roof *et al*., 1997), reduces brain oedema following TBI (Roof *et al*., 1996) and has anti-inflammatory properties (He *et al*., 2004a; Gibson *et al*., 2005). Best known is progesterone's ability to increase GABAergic activity, resulting in decreased neuronal excitability and consequent protection from excitotoxicity. It is likely that progesterone increases GABAergic activity indirectly, through metabolites that potentiate

Correspondence: Dr Paco Herson, as above. hersonp@ohsu.edu.

$GABA_A$ receptor activity. Progesterone is readily metabolised in the brain to 5α-dihydroprogesterone (DHP) by 5α-reductase, then further reduced to 3α,5α-tetrahyroprogesterone (ALLO) by the enzyme 3α hydroxysteroid reductase (for reviews see Celotti *et al*., 1992; Mellon & Vaudry, 2001; Stoffel-Wagner, 2001; Belelli & Lambert, 2005). While there is little direct evidence that progesterone's protective actions are dependent on its metabolism, (Allopregnanolone; ALLO) has been demonstrated to reduce ischemic damage and decrease glutamate excitotoxicity *in vitro* and *in vivo* (Lockhart *et al*., 2002; Ciriza *et al*., 2004, 2006; He *et al*., 2004b). Furthermore, a recent report indicates that ALLO is a more potent neuroprotectant than its parent compound in the middle cerebral artery occlusion model of transient ischemia, emphasizing that progesterone could act through its conversion to ALLO (Sayeed *et al*., 2006).

It has long been recognised that cerebellar Purkinje cells (PCs) are particularly sensitive to ischemic damage *in vivo* (Pulsinelli *et al*., 1982; Sato *et al*., 1990; Horn & Schlote, 1992; Brasko *et al*., 1995; Fonnum & Lock, 2000). The cellular mechanism and functional significance of this observation remains understudied. However, these cells are well-positioned neurochemically and synaptically to be vulnerable to ischemic challenges. PCs are the largest neurons in the cerebellar cortex and receive inhibitory inputs from Golgi, basket and stellate cells. PCs also receive excitatory inputs from granule cells (parallel fibers) and the inferior olive (climbing fibers). The enormity of excitatory input from both climbing and parallel fibers may make PCs susceptible to glutamate excitotoxicity through excessive activation of Ca^{2+}-permeable AMPA receptors (Brorson *et al*., 1994; Welsh *et al*., 2002; Slemmer *et al*., 2005). Accordingly, the AMPA receptor antagonist NBQX protects PCs after ischemia *in vivo* (Brasko *et al*., 1995). Elimination of climbing fiber input from the inferior olive also protects PCs from ischemic damage (Welsh *et al*., 2002). In addition, PCs receive large inhibitory inputs from interneurons. Therefore, the cerebellar cortex represents an ideal network in which to assess the effects of ischemia-induced excitotoxicity and the neuroprotective potential of compounds that potentiate GABAergic neurotransmission.

Using a novel PC culture system that preserves intact excitatory and inhibitory signalling (Gruol, 1983; Hirano *et al*., 1986; Hirano & Kasono, 1993), we set out to evaluate cerebellar PCs during oxygen and glucose deprivation (OGD) as a means of simulating cerebellar ischemia and testing the potential for GABAergic progesterone metabolites to protect these cells. We tested the hypothesis that progesterone's protective actions require its metabolism to ALLO and potentiation of $GABA_A$ receptors. Because recent reports suggest that male and female cells respond differently to toxic stimuli and to OGD (Liu *et al*., 2006; Du *et al*., 2004), the current study uniquely employed sex-specific neuronal cultures to evaluate whether genetic sex influences outcome and mechanisms of progesterone neuroprotection.

Materials and methods

Sex-specific cerebellar cultures

Neurons were cultured from the cerebellum of embryonic day (E)18 Sprague–Dawley rats in accordance with National Institute of Health guidelines and experimental protocols approved by the institutional IACUC committee. Male and female fetuses were identified as described previously (Liu *et al*., 2006). Briefly, embryos were sexed by visual inspection of the genital papilla, the presence or absence of testicular artery and sex chords and by measurement of the ano-genital space (larger in male). Polymerase chain reaction for the presence or absence of the Y-chromosome gene *sry* has been performed previously to verify 100% agreement with visual identification (Liu *et al*., 2006). Culture techniques were as previously described (Linden *et al*., 1991), briefly described here. Timed-pregnant Sprague–Dawley rats were killed with CO_2 and E18 fetuses removed by caesarean section and placed in ice-cold Hank's saline. Cerebelli were dissected and chopped into ~1 mm pieces before being digested for 30 min in

trypsin at 37 °C in Hank's saline. Digestion was halted by the addition of 10% fetal calf serum. The digested pieces were then triturated and filtered through a 70-μm nylon mesh. The cell suspension was then spun at 500 ***g*** and resuspended in neurobasal + B27 supplements (Invitrogen, Carlsbad, CA, USA). This culture medium contained no oestrogens and low-n_M progesterone. Cells were plated on 12-mm round coverslips coated with poly $_L$-lysine in 24-well culture dishes and maintained in a humidified atmosphere. Half the medium was replaced with fresh medium every 3–4 days. PCs are easily identified by their unique morphology (the largest neuron in the cerebellar cortex) and their immunoreactivity to the calcium binding protein calbindin; see Fig. 2 and Baimbridge & Miller (1982). Each culture yields a total of 18–24 coverslips of each gender with 30–50 PCs per coverslip. PCs represent < 5% of the neurons in culture, with the small non-PC neurons being a mixture of excitatory granule cells and inhibitory interneurons such as stellate and basket cells.

Immunocytochemistry

At 11–14 days *in vitro* (DIV) the growth medium was removed from coverslips and cerebellar neurons were washed with phosphate-buffered saline (PBS) immediately prior to being incubated for 20 min in freshly prepared 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA). Cells were then washed with PBS and incubated in asolution of PBS containing 0.2% Triton X-100 for 1 h in order to permeabilize the cells. Following three 10-min washes with PBS, cells were incubated for 1 h in blocking solution containing 5% goat serum, then incubated with a 1 : 1000 dilution of anticalbindin antibody (diluted in blocking solution) for 1 h at room temperature (Sigma monoclonal antibody C28K). Following four 10-min washes with PBS, cells were incubated with a 1 : 500 dilution of Cy3-conjugated goat antimouse IgG secondary antibody (Jackson Immuno, West Grove, PA, USA) for 1 h at room temperature before being washed and stained for terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labelling (TUNEL; see below) according to the manufacturer's instructions (Roche, Basel, Switzerland), and finally mounted on slides with Prolong antifade reagent (Molecular Probes, Eugene, OR, USA). Fluorescence imaging was performed on an upright Leica DMIRE2 microscope using Open Laboratory software (Improvision, Lexington, MA, USA) to capture and analyse images.

OGD and cell viability

OGD was induced by replacing the sex-steroid-free culture media with glucose-free deoxygenated saline (described below) solution and placing the cultures in an anaerobic incubator containing 5% CO_2 and 95% N_2 at 37 °C (Coy Laboratories Products, Grass Lake, MI, USA; contains catalyst ensuring oxygen levels < 1 p.p.m.) for 60–120 min and then returned to normoxia and normal culture media. We used TUNEL reactions to identify cells with fragmented DNA (Roche, Basel, Switzerland). It is important to note that the TUNEL assay does not distinguish among cell death mechanisms (necrosis or apoptosis); however, it is useful for detecting damaged cells with light or fluorescence microscopy at an early time point. Cell viability was assessed by visual inspection of damaged PCs stained with TUNEL and double-labelled with anti-C28K antibody to evaluate PC damage. Data are presented as percentages of TUNEL-positive PCs (TUNEL- and C28K-positive cells / total C28K-positive cells) on two coverslips from the same culture. The experimenter counted > 75 cells per experiment and was blinded to the exposure condition of each slide.

Electrophysiology

After 12–14 DIV, cultures were transferred to a recording chamber mounted on the stage of an inverted microscope equipped with a harmonic component system (Leica DM IL, Houston, TX, USA). PCs were selected by their unique morphology, large soma and prominent dendritic arbor. Whole-cell voltage-clamp experiments were made from the somas of PCs using an

Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA) interfaced to a Dell computer (Dell, Round Rock, TX, USA). Data were collected at a sample frequency of 20 kHz and analysed using pCLAMP9 software (Axon Instruments). Electrodes pulled from borosilicate glass capillaries, with inner filament, had resistances of 1–3 MΩ when filled with the internal solution (described below). Whole-cell capacitance and series resistance (60–80%) were electronically compensated. Spontaneous event detection was performed using a threshold crossing routine on 3-min sections of data. The total number of events observed in each 3-min section of data was used to calculate frequency. Twenty-five inhibitory postsynaptic currents (IPSCs) or excitatory postsynaptic currents (EPSCs) from each cell were randomly chosen and fitted with a sum of two or three exponentials to determine the average rise and decay time constants.

Solutions and drugs

The composition of the bathing saline solution was (in mm): NaCl, 140; KCl, 5; $MgCl_2$, 0.8; $CaCl_2$, 1; HEPES, 10; and glucose, 10; pH 7.35 with NaOH. PCs were recorded using an internal pipette solution consisting of (in mM): CsCl, 140; EGTA, 1; HEPES, 10; $MgCl_2$, 1; MgATP, 5; pH 7.3 with CsOH. CNQX and picrotoxin were obtained from Tocris (Ellisville, MO, USA). Allopregnanolone and finasteride were obtained from Sigma-Aldrich (St Louis, MO, USA) and progesterone was obtained from American Pharmaceutical Partners (Schaumberg, IL, USA). CNQX was dissolved in DMSO at a stock concentration of 50 mM. Picrotoxin was dissolved in EtOH at a stock concentration of 100 mM. Allopregnanolone was dissolved in DMSO at a stock concentration of 10 mm. Finasteride was dissolved in DMSO at a stock concentration of 10 mm.

Data analysis and statistics

Analysis of cell viability following OGD and treatment were performed blind; the identity of the treatment group was concealed and coded by a separate investigator. Each *n* represents a separate experiment performed on cultured cells obtained from a different pregnant rat. All data are presented as mean ± SEM. Statistical significance was determined with Student's *t*-test.

Results

Cultured PCs

Whole-cell voltage-clamp recordings obtained from cerebellar PCs contained inward spontaneous synaptic events corresponding to a mixture of inhibitory (GABAergic) and excitatory (glutamatergic) synaptic currents, in agreement with previous reports (Gruol, 1983; Hirano *et al*., 1986; Hirano & Kasono, 1993). The application of the $GABA_A$ receptor antagonist picrotoxin (100 μM) eliminated GABA-mediated currents and isolated glutamatergic spontaneous excitatory postsynaptic currents (sEPSCs). The complete inhibition of synaptic currents by the additional presence of CNQX confirmed their identity as AMPA receptor-mediated EPSCs (Fig. 1). A high degree of variability in the frequency and temporal pattern of sEPSCs was observed. In four out of six cells bursts of high-frequency sEPSCs (> 20 Hz) with lower frequency activity between bursts (6–12 Hz) were observed. Isolated sEPSCs were analysed to determine amplitude and kinetics. Spontaneous EPSCs had a mean amplitude of 27.6 ± 7.0 pA and exhibited rapid decay kinetics (τ_{off} = 5.1 ± 1.2 ms). Application of CNQX (5 lm) eliminated AMPA-mediated currents and isolated GABAergic spontaneous inhibitory postsynaptic currents (sIPSCs). In contrast to sEPSCs, sIPSCs were more regular in their temporal pattern. The average frequency of sIPSCs was 6.4 ± 1.5 Hz (*n* = 4), with a mean amplitude of 74.3 ± 11.5 pA (*n* = 4). Consistent with sIPSCs recorded from PCs in cerebellar brain slices (Konnerth *et al*., 1990; Southan & Robertson, 1998), the sIPSCs recorded from cultured PCs had a decay that was best fitted with a double exponential (τ_1 = 4.8 ± 2.1 ms and

τ_2 = 17.5 ± 2.4 ms). These data clearly demonstrate the presence of intact excitatory and inhibitory synapses onto cultured PCs, making this a good model for studying cerebellar responses to OGD in the context of an intact synaptic network.

PC sensitivity to ischemia and reperfusion

Experiments were performed using cerebellar cultures to assess the differential sensitivity of PCs to OGD. TUNEL reactions were used to identify cells with fragmented DNA. Cultures were treated with OGD for 2 h and cell viability was assessed 3 h after returning to normoxia (3 h reoxygenation). PCs were labelled with anticalbindin antibody (C28K; Fig. 2, Red) and damage assessed by fluorescent TUNEL staining to identify cells with DNA damage (Fig. 2, Green), with damaged cells appearing yellow in the merged image (Fig. 2, Merge). PCs were damaged to a significantly greater extent following OGD (69.4 ± 3.7% TUNEL-positive; n = 6) than non-PCs (49.7 ± 3.4%; n = 6; $P < 0.05$) in culture (Fig. 3a). Therefore, this study has focused on the response of PCs to OGD.

Experiments were performed on cultures from male and female embryos grown in the absence of background sex steroids in order to assess the effect of genetic sex on sensitivity of PCs to OGD. No sex difference in baseline sensitivity to OGD was observed (Fig. 3b). Significant PC damage was observed in both male cultures (49.9 ± 11.2% TUNEL-positive PCs, compared to 14.1 ± 2.3% control; n = 6) and female cultures (45.2 ± 12.9% TUNEL-positive PCs, compared to 17.1 ± 6.0% control; n = 6) following 1 h OGD. Similarly, 2 h OGD caused significant and more consistent levels of damage in both male (69.5 ± 2.3%; n = 38; $P < 0.05$) and female (64.8 ± 2.3%; n = 22; $P < 0.05$) cultures (Fig. 3), and therefore all further experiments were performed using 2 h OGD.

Progesterone neuroprotection of PCs

Progesterone has recently been demonstrated to be protective in animal models of cerebral ischemia (for review see Stein & Hoffman, 2003); however, little is known of the neuroprotective potential of progesterone in the cerebellum. The application of a pharmacological dose of progesterone (1 μM) 15 min prior to OGD, and maintained throughout OGD and reoxygenation, resulted in significant protection of PCs from OGD, independent of sex, decreasing damage from 58.4 ± 6.8% to 21.3 ± 4.1% (n = 7; $P < 0.05$) in male cultures and from 61.9 ± 4.9% to 23.8 ± 5.6% (n = 5; $P < 0.05$) in female cultures. Furthermore, there was a dose-dependent protection of PCs by progesterone (Fig. 4), with significant protection observed at all concentrations tested in female cultures and significant protection observed at concentrations ≥ 30 nm in male cells.

In order to test the hypothesis that progesterone is neuroprotective through interaction with $GABA_A$ receptors, the $GABA_A$ receptor antagonist picrotoxin was applied to cerebellar cultures. The additional presence of 100 μM picrotoxin (a concentration known to completely inhibit $GABA_A$ receptor activity in these cells; see Fig. 1) prevented the protection afforded by progesterone to male PCs, increasing the damage from 24.4 ± 6.8% in progesterone alone (1 μM) to 45.6 ± 5.4% in progesterone + picrotoxin (Fig. 5; n = 4; $P < 0.05$). Picrotoxin did not effect PC survival on its own (data not shown). Similar data were obtained in female cultures (data not shown). Progesterone does not directly modulate the activity of $GABA_A$ receptors, while the neuroactive metabolite ALLO is among the most potent positive modulators of $GABA_A$ receptor activity. Therefore, the ability of ALLO to protect PCs from OGD-induced damage was tested. Similar to progesterone, ALLO was protective at concentrations as low as 10 nM in both male and female cultures (Fig. 5) and picrotoxin reversed the protection afforded by high levels of ALLO (1 μM), increasing the damage from 27.9 ± 6.7% to 62.5 ± 9.2% in male cultures (Fig. 5; n = 4; $P < 0.05$). Finally, the 5α-reductase inhibitor finasteride was used to determine whether progesterone protection of PCs requires its conversion to an active

metabolite. In agreement with this hypothesis, coapplication of 10 μM finasteride reversed the protection afforded by pharmacological doses (1 μM) of progesterone (Fig. 6). Importantly, finasteride had no effect on the protection provided by ALLO (Fig. 6).

The ability of both progesterone and ALLO to protect PCs from OGD-induced damage when applied after OGD was examined. In male cultures, 300 nM progesterone and 300 nM ALLO caused significant protection when applied concurrently with reoxygenation (decreased damage from 70.6 ± 6.5% to 42.4 ± 7.6% and 35.9 ± 3.7% for progesterone and ALLO, respectively; Fig. 7; $n = 4$). Similar results were found for female cultures, with 300 nM progesterone decreasing damage from 69.8 ± 6.5% to 48.3 ± 10.5% ($n = 4$; $P < 0.1$) and ALLO decreasing damage from 69.8 ± 6.5% to 36.5 ± 14.6% ($n = 4$; $P < 0.05$) when applied immediately following OGD. These data indicate that progesterone and ALLO are able to protect PCs when applied following the ischemic event, suggesting a potential for therapeutic benefit.

Discussion

In this study cerebellar cultures obtained from male and female embryos were used to examine the sensitivity of male and female PCs to OGD and to test whether progesterone protects PCs from OGD through active metabolites that increase GABAergic activity. The data indicate that PCs are more sensitive than other cerebellar neurons to experimental ischemia (OGD), but that there is no difference in the sensitivity of male and female PCs to OGD. This study also demonstrated that physiological concentrations of progesterone protect male and female PCs from OGD and that the progesterone protection was reversed by the inhibition of $GABA_A$ receptors. Further experiments revealed that progesterone neuroprotection is probably mediated through a metabolite that directly potentiates $GABA_A$ receptor activity.

The cerebellum is well suited as a model for studying cell death, because damage leads to easily recognised phenotypes such as ataxia and impaired motor coordination (Trouillas *et al*., 1997). As a result, a large number of cerebellar disorders have been identified in humans and mice, and all involve PC compromise (for review see Sarna & Hawkes, 2003). Because the efferent pathway from PCs to deep cerebellar nuclei is the sole output of the cerebellar cortex, PC loss results in a functional lesion of the cerebellum. The anatomy and neuronal circuitry of the cerebellum is well defined and PCs are the largest neuron in the cerebellar cortex, receiving excitatory input from granule cells and inhibitory inputs from Golgi, basket and stellate cells. In agreement with earlier findings, we demonstrated that cultured cerebellar PCs receive robust excitatory inputs from granule cells as well as inhibitory inputs from interneurons (Gruol, 1983; Hirano *et al*., 1986; Hirano & Kasono, 1993). This mixed cerebellar neuron culture offers important advantages over typical homogenous neuronal cultures. First, PCs are known to be hypersensitive to stress and ischemia–hypoxia, but relatively few studies have attempted to elucidate the mechanism behind their sensitivity or potential therapeutic interventions. Secondly, the presence of multiple cerebellar neuronal cell types allows for the generation of a network *in vitro* containing both inhibitory and excitatory neurons, enabling us to assess the effects of ischemia on the balance between excitatory and inhibitory transmission and the enhancement of endogenous inhibition as a mode of neuroprotection. Consistent with *in vivo* reports, this report demonstrated that cultured PCs are the most sensitive neuron to OGD in the cerebellum. To our knowledge this is the first neuronal culture model demonstrating PC sensitivity to ischemia–reperfusion.

There is emerging evidence that cellular responses to stresses causing cell death are sex-specific, independent of exposure to sex steroids (Lieb *et al*., 1995; Du *et al*., 2004; Liu *et al*., 2004, 2006). However, we did not observe any difference in sensitivity to OGD between male and female PCs. This may indicate that the neuronal response to such complex insults as

ischemia is not sex-specific, in contrast to that observed for non-neuronal brain cells (Liu *et al*., 2006). Alternatively, there may be regional differences, such that primary cerebellar neurons do not respond in a sexually dimorphic manner, but neurons from other brain regions may. Despite the negative result reported here, this remains an interesting new topic that merits further investigation.

Progesterone has been demonstrated to be neuroprotective in a variety of experimental animal models of focal and global ischemia, including TBI (Stein & Hoffman, 2003), middle cerebral artery occlusion (Roof & Hall, 2000; Murphy *et al*., 2002; Gibson & Murphy, 2004) and cardiac arrest (Gonzalez-Vidal *et al*., 1998; Cervantes *et al*., 2002). However, the mechanism of neuroprotection remains in question. The concentration of progesterone in the brain is thought to closely follow circulating serum levels; therefore we tested the protection afforded by acute progesterone across a concentration range (10–200 n_M) consistent with the levels measured in rat serum (Butcher *et al*., 1974; Nequin *et al*., 1979) as well as pharmacological doses up to 1 μ_M. Results in the present study indicate that concentrations of progesterone consistent with low physiological levels in the rat (10 n_M) protect both male and female cerebellar PCs in culture against OGD. Progesterone is readily metabolised into compounds such as ALLO which directly interact with inhibitory $GABA_A$ ionotropic receptors (for reviews see Mellon & Vaudry, 2001; Stoffel-Wagner, 2001; Belelli & Lambert, 2005). In order to isolate the rapid nongenomic effects of progesterone, primary neuronal cultures were grown in the absence of sex steroids and progesterone was acutely applied immediately prior to exposure to OGD. Three separate experiments were performed to directly test the hypothesis that progesterone neuroprotection is mediated via an active metabolite that potentiates GABAergic neurotransmission. Firstly, inhibition of $GABA_A$ receptors with picrotoxin prevented the protection afforded by progesterone, indicating that $GABA_A$ receptor activity is required for progesterone neuroprotection. Secondly, the GABA-active progesterone metabolite ALLO protects PCs, and again neuroprotection was prevented by picrotoxin, indicating a central role for $GABA_A$ receptors in progesterone and ALLO neuroprotection. Finally, inhibition of progesterone metabolism with a 5α-reductase inhibitor (finasteride) reversed the protection afforded by progesterone while having no effect on ALLO-mediated neuroprotection. These data, taken together, indicate that the progesterone protection of cerebellar PCs is mediated through a GABA-active metabolite, probably ALLO, and that intact $GABA_A$ receptors are required for neuroprotection.

The conversion of progesterone to ALLO in the brain takes place predominantly within glial cells (Celotti *et al*., 1997). Glia perform a variety of functions that are critical for neuronal health, including glutamate uptake and release, water transport and hormone metabolism; they thus play a significant role in the response to ischemia (for review see Chen & Swanson, 2003). In particular, glial cells within the cerebellum appear to alter the response and sensitivity of neurons to ischemia and excitotoxicity (Beaman-Hall *et al*., 1998; Barakat *et al*., 2002; Welsh *et al*., 2002; Yamashita *et al*., 2006). Therefore, progesterone neuroprotection *in vivo* may involve interaction with glial cells, neurons or other cell types. The use of a pure neuronal culture in the present study demonstrates that progesterone neuroprotection in the cerebellum involves direct interaction with neurons. Importantly, cerebellar PCs are unique neurons that express the enzymes (5α-reductase and 3α-hydroxysteroid dehydrogenase) necessary to metabolise progesterone into ALLO (Tsutsui *et al*., 2003), allowing progesterone neuroprotection via interconversion to ALLO in the absence of supporting glial cells.

GABA is the predominant inhibitory neurotransmitter in the brain and is therefore an appealing target for counteracting the excessive excitatory stimulation observed following ischemia. While many studies have demonstrated protection with GABA modulators, it is important to note that multiple studies have failed to observe protection with similar compounds (for review see Schwartz-Bloom & Sah, 2001). Why then have so many studies failed to see a robust

protection following application of GABA-active molecules? The answer is probably in the detailed analysis of regional differences. That is, networks that have large inhibitory inputs are likely to be protected by GABA potentiation, while regions comprised of predominantly excitatory inputs will not benefit from such treatment. The enormity of the excitatory input from both climbing and parallel fibers may make cerebellar PCs particularly vulnerable to excitotoxicity. In agreement with this hypothesis, the AMPA receptor antagonist NBQX partially protects PCs from ischemia *in vivo* (Brasko *et al*., 1995). Similarly, we observed that the AMPA receptor antagonist CNQX afforded robust protection against OGD in our cultured PC model (data not shown). The cerebellum is also unique in the strength of inhibitory drive exhibited on the PC. For example, *in vivo* recordings demonstrate that the addition of the GABA agonist muscimol completely inhibits spontaneous PC activity (Caesar *et al*., 2003). Therefore, the cerebellum represents an ideal brain region in which to study the ability of inhibitory synaptic activity to suppress excitotoxicity. In addition, while the cerebellum has long been known to be a region of the brain particularly vulnerable to a variety of insults, it is relatively understudied. The data presented in this study indicate that progesterone, through its neuroactive metabolite ALLO, increases $GABA_A$ receptor activity and protects cultured PCs from OGD, suggesting that GABA modulators may hold therapeutic potential, at least as it pertains to the cerebellum. Therefore, it would be of interest to test the ability of progesterone to protect cerebellar PCs in *in vivo* models of cerebral ischemia and neurodegeneration involving the cerebellum.

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Abbreviations

ALLO	3α,5α-tetrahyroprogesterone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate
DHP	5α-dihydroprogesterone
E	embryonic day
EPSC	excitatory postsynaptic current
IPSC	inhibitory postsynaptic current
NBQX	2,3-dioxo-6-nitro-1,2,3,4- tetrahydrobenzo[f]quinoxaline-7-sulphonamide
OGD	oxygen and glucose deprivation
PBS	phosphate-buffered saline
PC	Purkinje cell
sEPSC	spontaneous EPSC
sIPSC	spontaneous IPSC
TBI	traumatic brain injury
TUNEL	terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling

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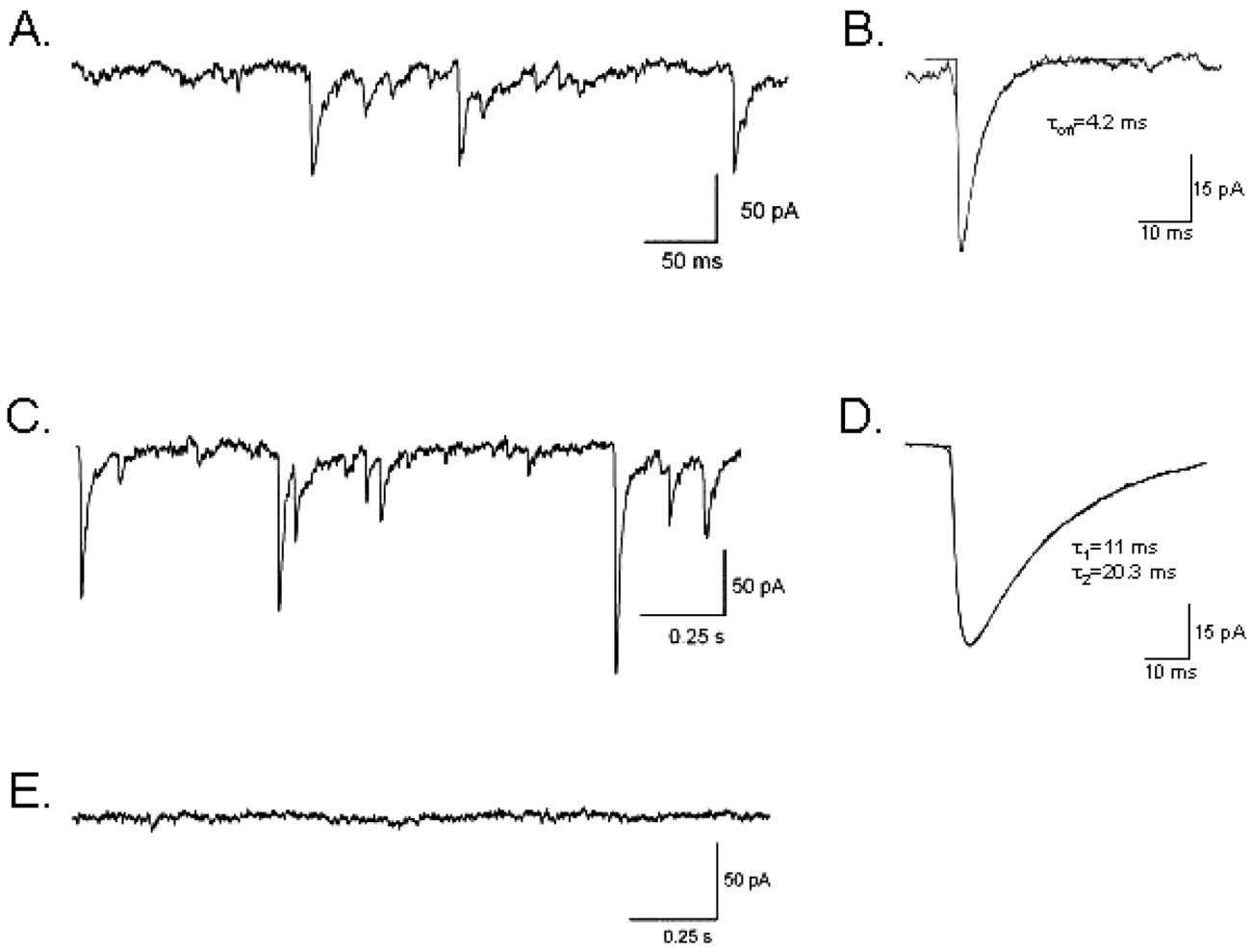

Fig. 1.

Cultured cerebellar PCs generate intact inhibitory and excitatory synapses. (A) Representative trace of sEPSCs recorded in the presence of 100 μM picrotoxin. (B) Average of multiple events fitted to determine rise and decay time constants. (C) Representative trace of spontaneous inhibitory postsynaptic currents recorded in the presence of 5 μM CNQX. Note different timescale from trace A. (D) Average of multiple events fitted to determine rise and decay time constants. (E) Trace recorded in the presence of both picrotoxin and CNQX.

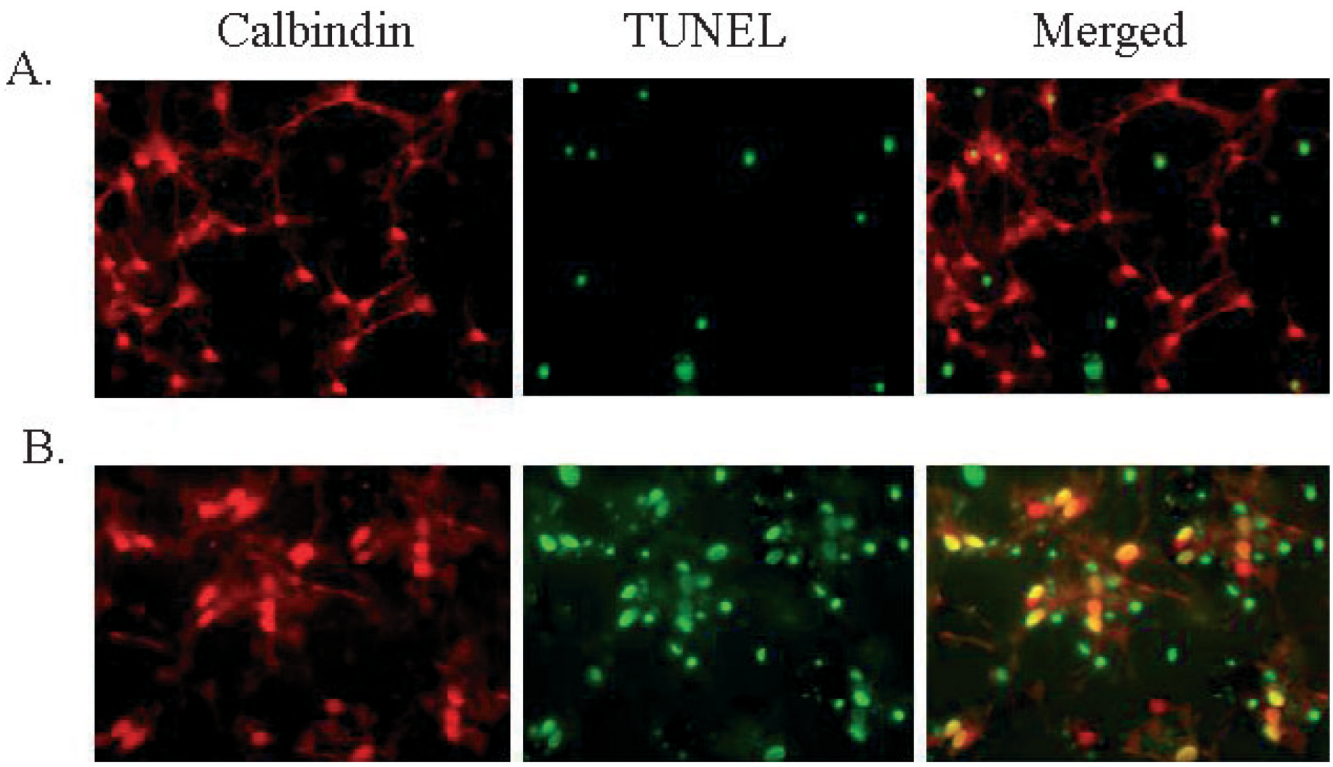

Fig. 2.

Exposure of cultured cerebellar PCs to 2 h OGD resulted in significant cell death as indicated by TUNEL staining. (A) Representative images of control cultures double-stained with anticalbindin (red) to identify PCs and TUNEL (green) to indicate DNA damage. (B) Representative images of the significant damage observed in PCs following 2 h OGD, as indicated by the high number of yellow (merged) cells.

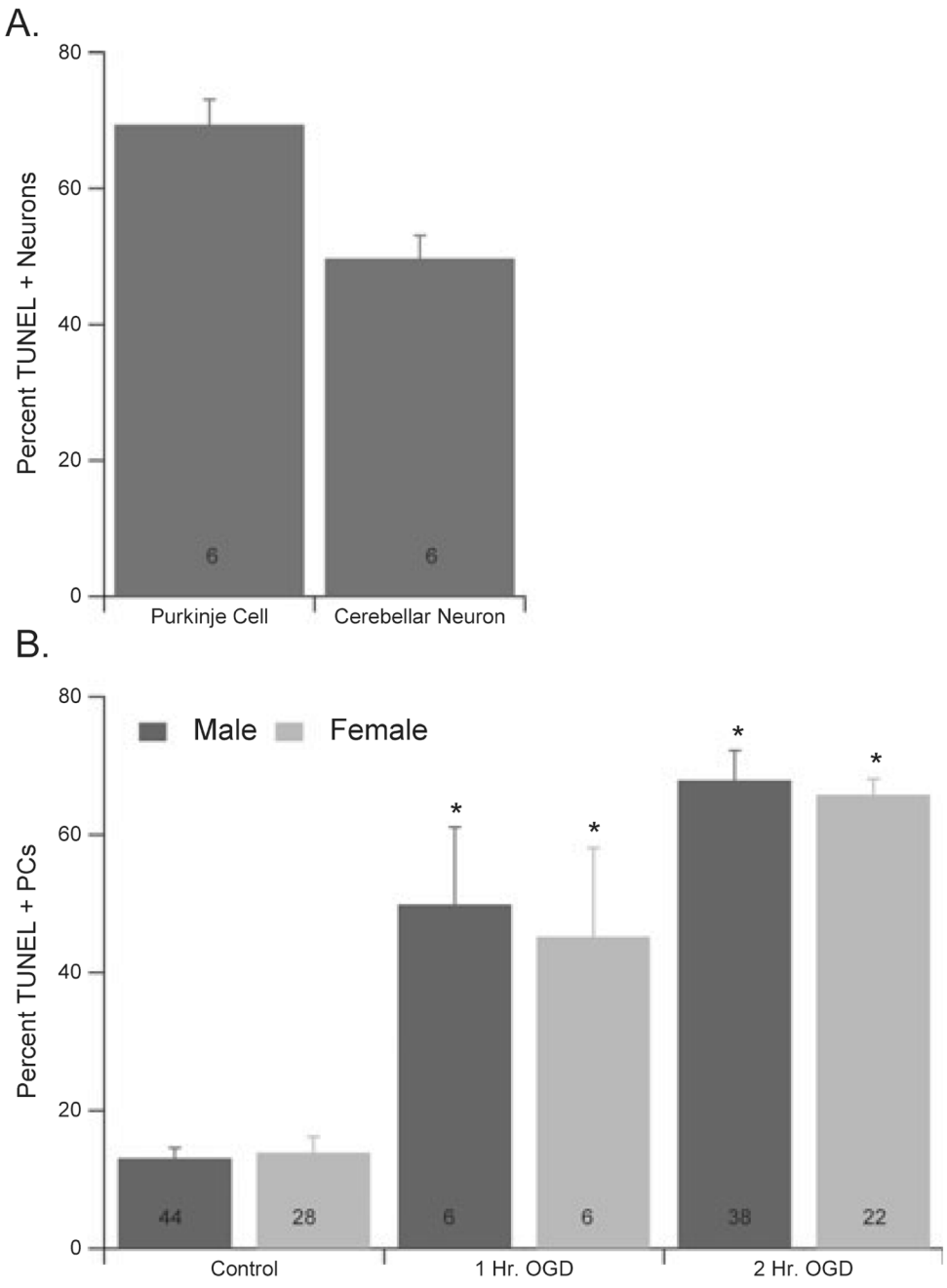

Fig. 3.

Male and female PCs were equally sensitive to OGD. (A) Quantification of the differential sensitivity of cerebellar neurons exposed to 2 h of OGD, indicating that PCs are significantly more sensitive to OGD than non-PC cerebellar neurons. (B) Quantification of damage observed in male and female PCs exposed to 1 or 2 h of OGD. The percentage of TUNEL-positive PCs was assessed following 3 h reoxygenation. Numbers in each bar represent number of experiments performed, each on a different culture. Data are mean ± SEM. **P* < 0.05 vs. vehicle-treated cells (2 h OGD).

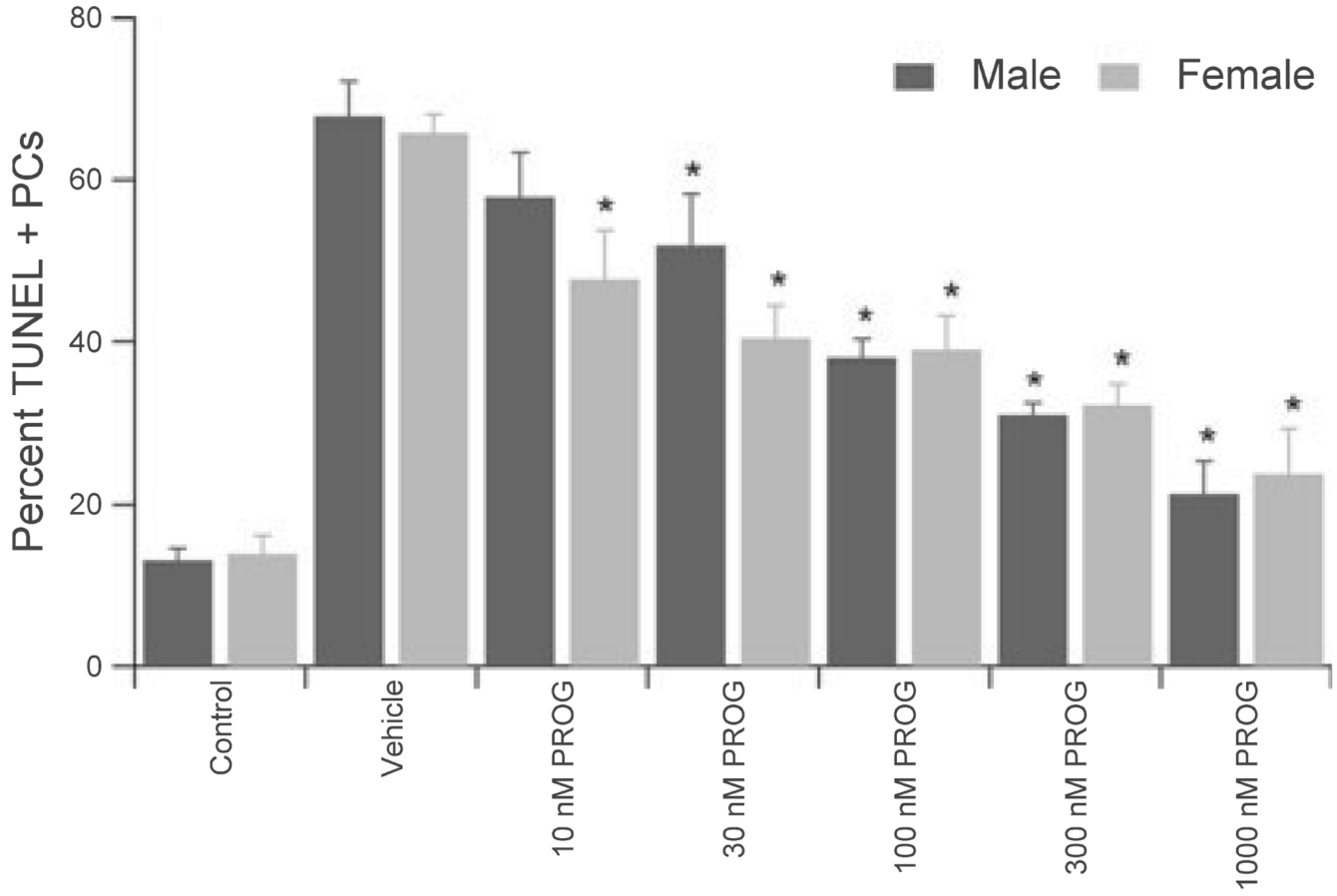

Fig. 4.

Progesterone protected PCs in a concentration-dependent manner. Male and female PCs were exposed to the various concentrations of progesterone for 15 min prior to OGD and maintained throughout reoxygenation. Data are mean ± SEM. **P* < 0.05 vs. vehicle-treated cells (2 h OGD) from the same sex.

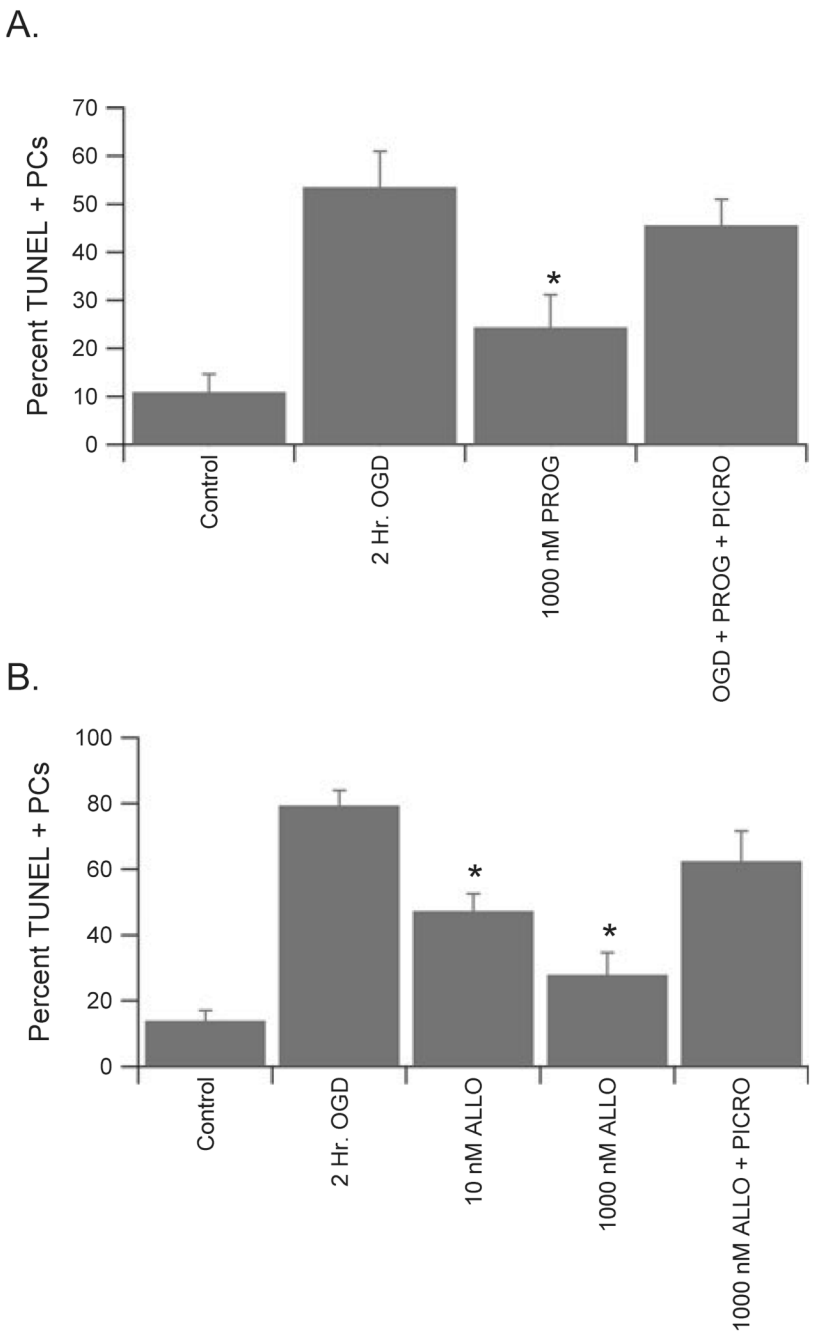

Fig. 5.

Progesterone and its metabolite ALLO protected PCs via activation of $GABA_A$ receptors. (A) Quantification of the neuroprotective effect of exposure to 1 μM progesterone (PROG) for 15 min prior to OGD and maintained throughout reoxygenation. The additional presence of 100 μM picrotoxin prevented the protection afforded by progesterone. (B) Quantification of the neuroprotective effect of exposure to 1 μM ALLO for 15 min prior to OGD and maintained throughout reoxygenation. The additional presence of 100 μM picrotoxin prevented the protection afforded by ALLO. Data are mean ± SEM. **P* < 0.05 vs. vehicle-treated cells (2 h OGD).

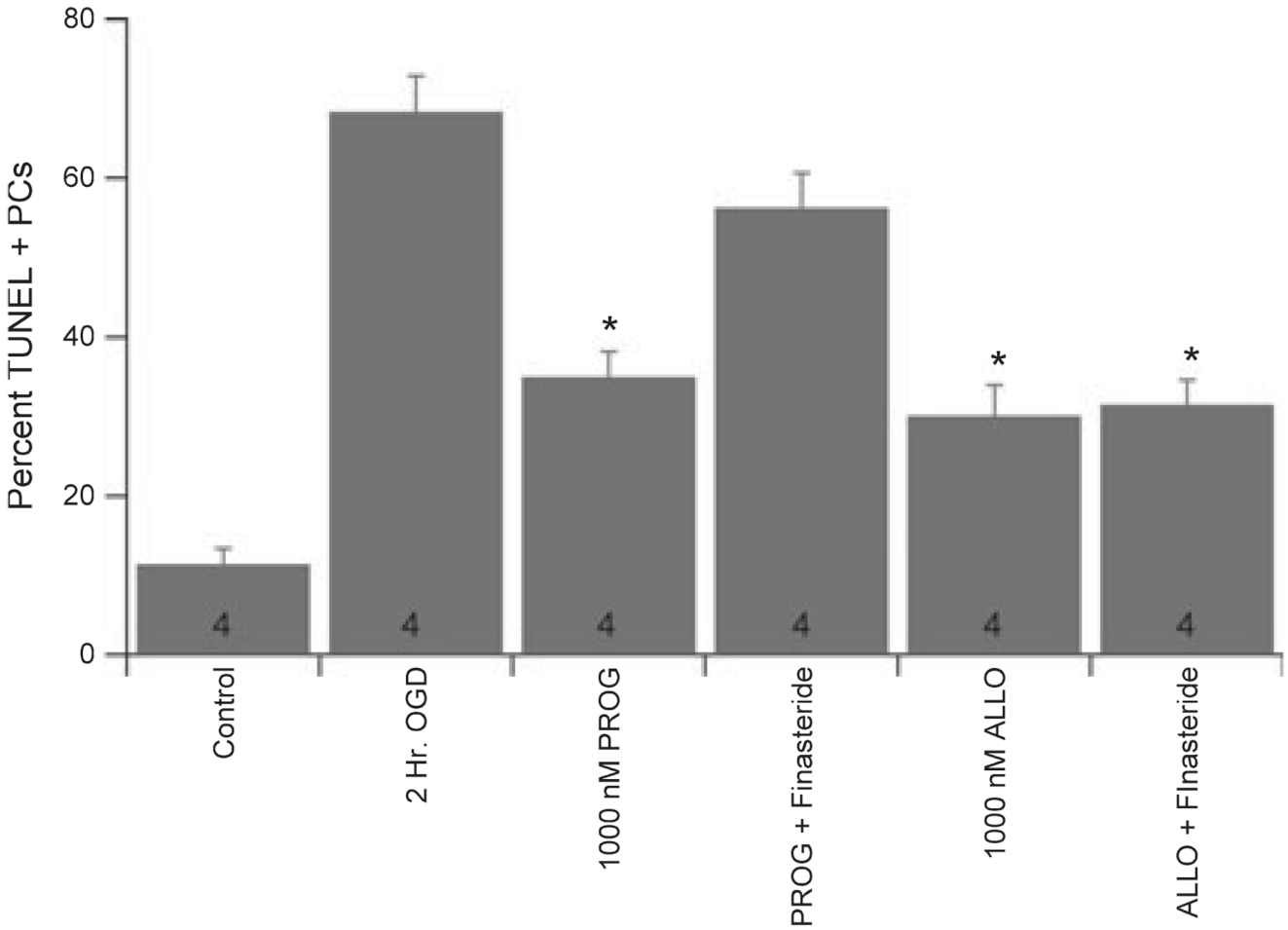

Fig. 6.

Progesterone neuroprotection required its metabolism. Quantification of the ability of finasteride to prevent progesterone neuroprotection. PCs were preincubated with 10 μM finasteride for 15 min, then exposed to either 1 μM progesterone or ALLO for another 15 min prior to OGD and maintained throughout reperfusion. Data are mean ± SEM. **P* < 0.05 vs. vehicle-treated cells (2 h OGD).

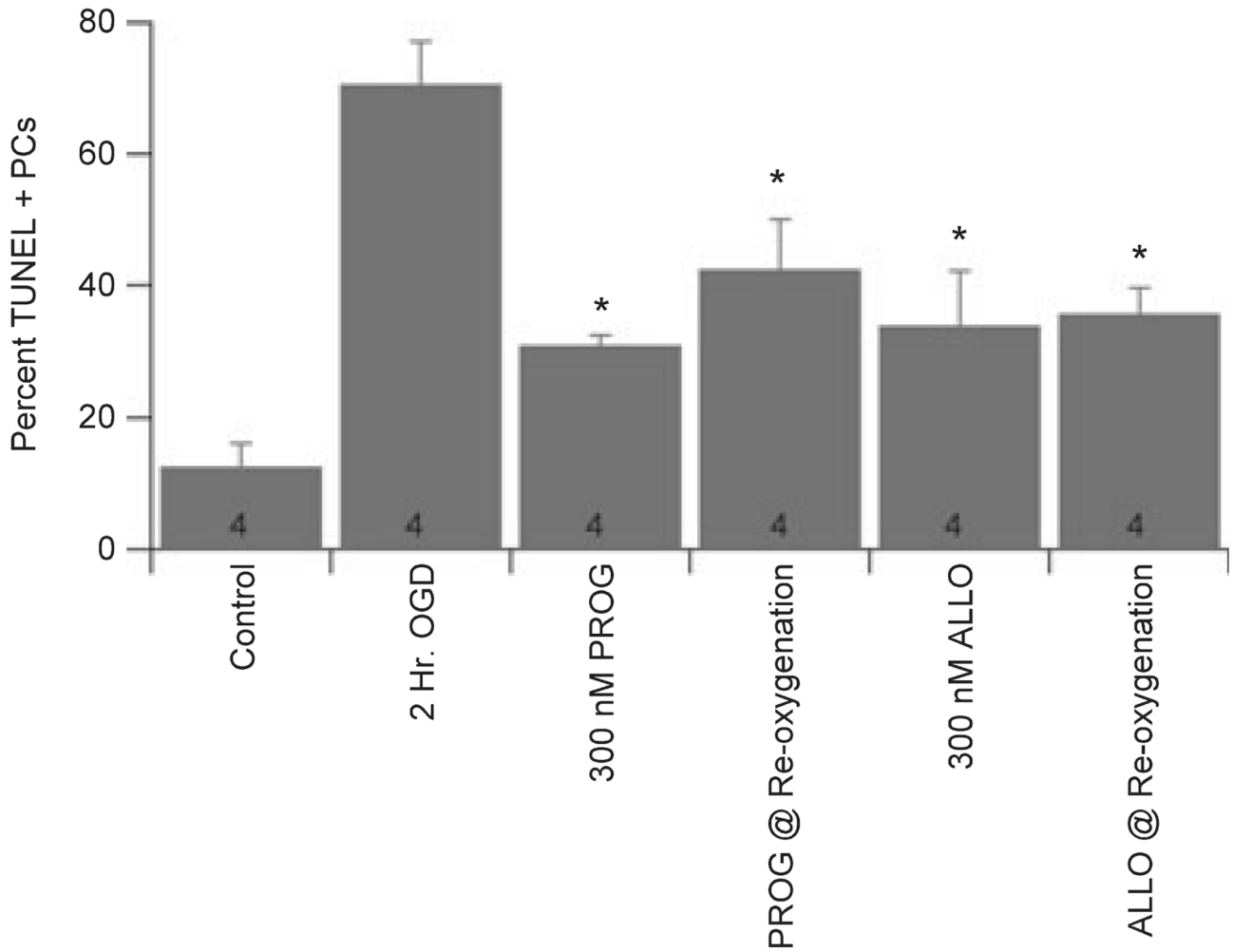

Fig. 7.

Progesterone and ALLO effectively protected PCs when applied after OGD. Progesterone and ALLO were applied to PC cultures concurrent with reoxygenation (PROG or ALLO @reoxygenation). Data are mean ± SEM. **P* < 0.05 vs. vehicle-treated cells (2 h OGD).