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Research Report

Allopregnanolone effects on astrogliosis induced by hypoxia in organotypic cultures of striatum, hippocampus, and neocortex

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ABSTRACT

Perinatal asphyxia occurs in approximately 0.3% full-term newborn babies, and this percentage has not decreased despite medical advances. There are now evidences indicating that neurosteroids are important in neurodevelopment showing neuroprotective effects. We studied the potential protective effect of allopregnanolone (Allo) in vitro using organotypic cultures from neocortex, striatum, and hippocampus. Immunocytochemistry and confocal microscopy showed an increase of the glial fibrillary acidic protein (GFAP) signal in the studied brain areas after hypoxia. Western blot studies supported these results (hippocampus, 193%; neocortex, 306%; and striatum, 231%). Twenty-four-hour pretreatment with Allo showed different effects at the brain areas studied. In the hippocampus and the neocortex, 24-h pretreatment with Allo 5×10^{-6} M showed to be neuroprotective as there was a significant decrease of the GFAP signal compared to control cultures exposed to hypoxia. Pretreatment with 5×10^{-8} M Allo attenuated the astrogliosis response in the hippocampus and the neocortex in a nonsignificant way. Allo pretreatment at all doses did not show to affect the astrogliosis triggered by hypoxia in the striatum. Cell survival was analyzed by measuring LDH. After 1 h of hypoxia, all cultures showed a nonsignificant increase of LDH, which was greater after 24 h of hypoxia (hippocampus, 180%; striatum–cortex co-cultures, 140%). LDH levels have no changes by Allo pretreatment before hypoxia. Conclusion: 24 h pretreatment with 5×10^{-6} M of Allo does not change neuronal viability but it prevents astrogliosis induced by hypoxia in the hippocampus and the neocortex.

1. Introduction

The neurosteroid 3α-hydroxy-5α-pregnan-20-one, allopregnanolone (Allo) is synthesized by the reduction of progesterone via the enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase in the adrenal medulla, ovary, and brain. Allo is a potent endogenous steroid that rapidly affects the excitability of neurons and glia cells through direct modulation of the

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Abbreviations: Allo, allopregnanolone; GFAP, glial fibrillary acidic protein; NOS, nitric oxide synthase

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$GABA_A$ receptors activity exerting hypnotic/sedative, anxiolytic, anesthetic, and anticonvulsive properties (Crawley et al., 1986; Devaud et al., 1995; Lambert et al., 2003; Wieland et al., 1991). There are two neurosteroids binding sites in the $GABA_A$ receptor's transmembrane domains, one is responsible for potentiating $GABA_A$ responses whereas the other acts on direct receptor activation. Both sites have to be occupied to potentiate inhibitory GABAergic transmission (Hosie et al., 2006). The potency of neurosteroids may be regionally specific because their action results from the characteristics of the steroid and from the $GABA_A$ receptor subunit composition (Laurie et al., 1992). Allopregnanolone slows the rate of recovery of $GABA_A$ receptors from desensitization and it has been proposed that it also increases the rate of entry into fast desensitized states (Zhu and Vicini, 1997).

Accumulating evidence suggests the neuroprotective actions of Allo in a variety of experimental paradigms. Allo protects NMDA-induced excitotoxicity and apoptosis in neurons in vitro (Frank and Sagratella, 2000; Lockhart et al., 2002; Xilouri and Papazafiri, 2006) and it has positive effects on brain injury in vivo (Di Michele et al., 2000; Djebaili et al., 2005). Moreover, neuroprotective actions of Allo have been shown in hypoxia-induced brain injury. In pregnant sheep, Allo concentrations are high during fetal life and rise further in response to acute hypoxic stress as a protective mechanism to reduce excitotoxicity (Hirst et al., 2006). Besides perturbation of fetal brain Allo tonus affects the developing brain increasing the severity of hypoxia-induced brain injury (Yawno et al., 2007). In vitro studies report different effects of Allo upon hypoxia. In cortical primary cultures Allo shows no effect on neuronal survival (Marx et al., 2000) while it has neuroprotective effects in hippocampal and cerebellar cultures after hypoxia (Ardeshiri et al., 2006; Frank and Sagratella, 2000). Recent studies from our laboratory show that rats subjected to perinatal asphyxia present long-lasting biochemical and neurological changes in adulthood. Among the outcomes there are chronic cytoskeletal alterations and glia hyperthrophy (Cebral et al., 2006). Astrocytes play pivotal roles providing metabolic and trophic support to neurons (Aschner et al., 1999; Chiu and Kriegler, 1994; Dienel and Hertz, 2001). In response to injury, including cerebral ischemia, astrocytes proliferate and adopt a reactive phenotype expressing and secreting soluble factors that could have both beneficial and detrimental effects on injured brain (Araque, 2006; Ridet et al., 1997).

Here, we examined whether the gliosis response observed in a previous model of perinatal asphyxia were reproducibled in vitro, in order to evaluate the potential neuroprotective effect of Allo. The degree of astrogliosis triggered by hypoxia was assessed in organotypic cultures from hippocampus, neocortex, and striatum submitted to hypoxia. LDH levels were also measured as indicator of neuronal cell viability. The results from this study suggest that neurosteroids could have significant clinical relevance to disorders involving neuronal damage by hypoxic insults.

2. Results

Tissues exposure to hypoxia for 1 h produced an increase of GFAP labeling in hippocampus, neocortex, and striatum. The expression revealed by immunocytochemistry suggests an intense astroglial reaction triggered by asphyxia in these brain areas (Fig. 1A). Western blot studies supported these observations. Cultures subjected to hypoxia showed an increase in GFAP signal in the hippocampus (193±35%, $p< 0.01$), neocortex (306±63, $p< 0.05$), and the striatum (231±51%, $p< 0.05$) compared to control cultures under normoxia (Fig. 1B).

To evaluate the neuroprotective effect of Allo cultures were treated with different concentration (5×10^{-6}, 5×10^{-8}, and 5×10^{-10} M) for 24 h. Afterwards, half of the cultures were subjected to 1 h of hypoxia while the other half were kept under normoxic conditions. Allo treatment showed different effects depending on the doses and the brain areas evaluated. Allo 5×10^{-6} M significantly decreased hypoxia induced-astrogliosis in the hippocampus (40±12%; Fig. 2) and the neocortex (41±11%; Fig. 3). Twenty-four-hour pretreatment with Allo 5×10^{-8} M had a slight nonsignificant decreased of the GFAP signal in the hippocampus (14±6%) and the neocortex (23±5%) compared with vehicle-exposed controls. Pretreatment with Allo 5×10^{-10} M did not prevent astrogliosis in the hippocampus or the neocortex (Figs. 2 and 3, respectively). Pretreatment with Allo at any dose had no effects on GFAP immunostaining in the absence of hypoxia either in the hippocampus or the neocortex (Figs. 2 and 3, unfilled bars). In contrast to the effect in the hippocampus and neocortex, pretreatment with Allo at any dose studied did not attenuate the astrogliosis induced by hypoxia in the striatum (data not shown).

Finally, neuronal survival was estimated by measuring LDH released in the incubation media. After 1 h of hypoxia all cultures showed a nonsignificant increase of LDH, which was greater after 24 h of hypoxia (hippocampus, 180±28%; striatum–cortex co-cultures, 140±21%). Pretreatment with Allo 5×10^{-6} M before hypoxia did not prevent neuronal death (Fig. 4).

3. Discussion

This study demonstrates for the first time that Allo pretreatment down-regulate GFAP expression and thereby the gliosis reaction triggered by hypoxia in vitro. Our data show that pretreatment with Allo (5×10^{-6} M) has a protective effect on the gliosis induced by hypoxia at physiologically relevant concentrations in rat organotypic neocortical and hippocampal but not striatal cultures. These results are in agreement with previous studies showing that the treatment with Allo or its precursor progesterone reduce the accumulation of reactive astrocytes induced by cortical wound (Garcia-Estrada et al., 1993, 1999) and cortical impact head injury (Djebaili et al., 2005) in vivo.

The "astroglial reaction" has been extensively studied in many models (Petito et al., 1990). After a brain injury, the astrocytes participate actively in the glial reaction and exhibit a differential phenotype that has been called "reactive". They become hypertrophied when the number of intermediate filaments is increased and are more immunoreactive to GFAP. As neuronal nutrition and the maintenance of the oxygen supply are highly dependent on functioning astrocytes

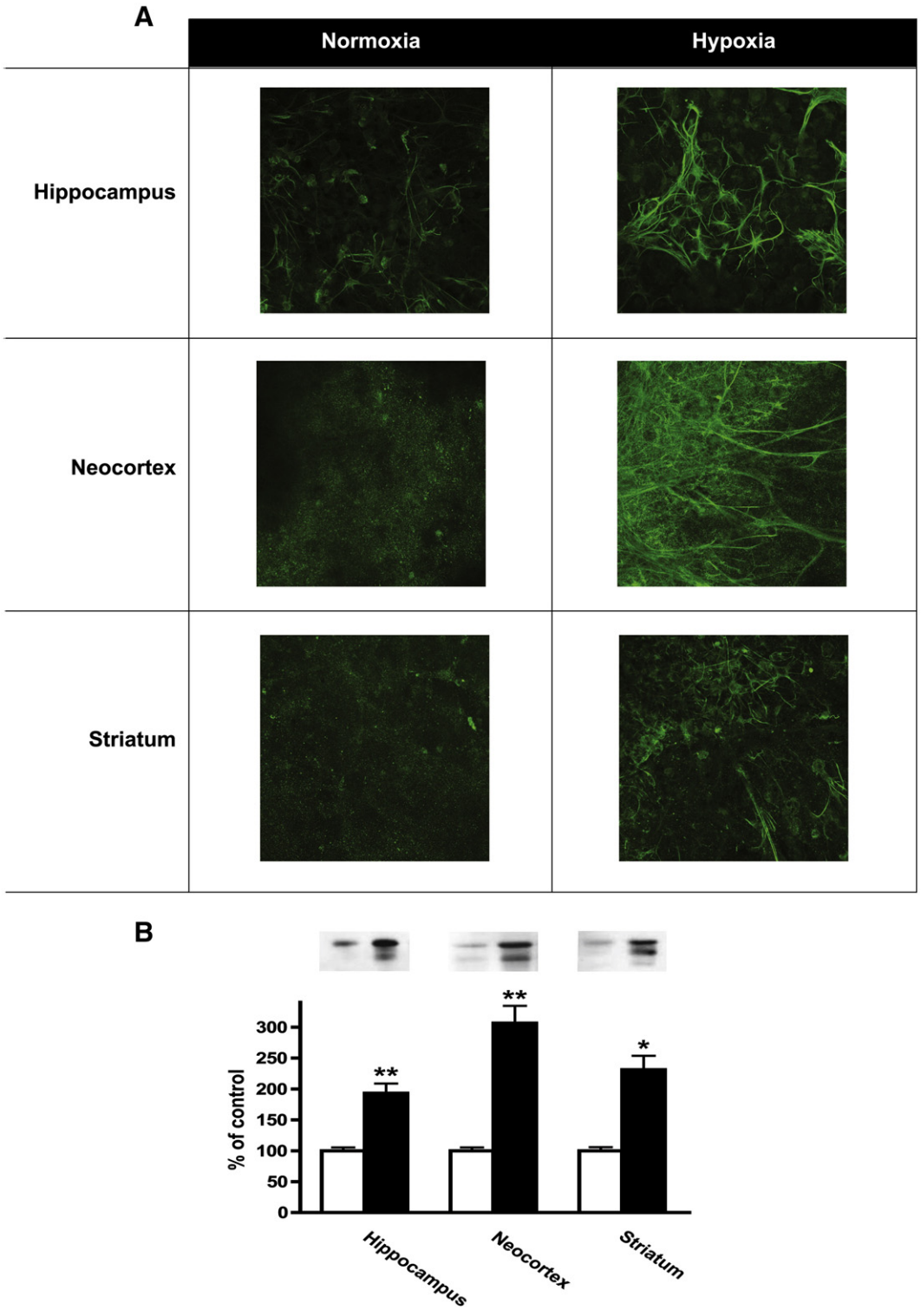

Fig. 1 – (A) Immunocytochemistry and confocal microscopy showed an increase of GFAP signal after hypoxia in the hippocampus, neocortex, and striatum organotypic cultures. (B) Western blot studies supported these results (hippocampus, 193%; neocortex, 306%; striatum, 231%, * $p < 0.05$, ** $p < 0.01$ Student's t-test; $n = 4$).

(Magistretti and Pellerin, 1996), the increase in reactive astrocytes leads to a nutritional imbalance that affects injured neurons most severely.

The mechanisms by which Allo attenuates gliosis following hypoxia are likely to be diverse. It is known that an anoxic insult increase extracellular glutamate release and promote excitotoxic cell death (Engidawork et al., 2001). N-metyl-D-aspartate (NMDA) receptor activation leads to neuronal NOS induction and NO synthesis (Cardenas et al., 2000; Loidl et al., 1998). The increase of NO production

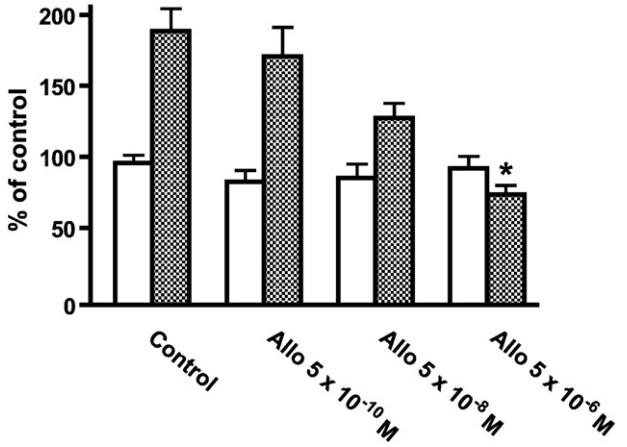

Fig. 2 – Effect of Allo on the astrogliosis induced by hypoxia in the hippocampus. Empty bars, cultures treated with Allo or vehicle under normoxia. Filled bars, treated cultures subjected to 1 h of hypoxia. Two-way ANOVA showed a significant effect by hypoxia [$F_{(1,60)}=32.8$ $p<0.001$] and by treatment [$F_{(3,60)}=8.6$ $p<0.001$]. Allo (5×10^{-6} M) treatment significantly decreased GFAP signal triggered by hypoxia in hippocampal cultures (40±12%), *$p<0.005$, Fisher PLSD test.

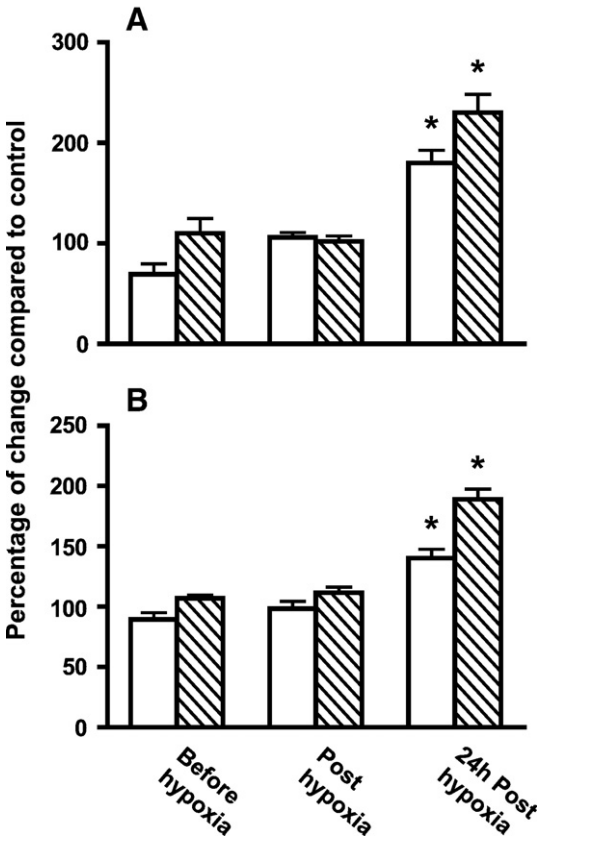

Fig. 4 – Neuronal survival was estimated by measuring LDH released in the incubation media. LDH activities are presented as ratio between LDH activity after treatment and LDH activity of control slice cultures kept in normoxic conditions at each time. Twenty-four hours after hypoxia, LDH was significantly increased in both hippocampal (A) and striatum–cortex (B) cultures. Filled bars, cultures subjected to hypoxia in the presence of Allo (5×10^{-6} M). Unfilled bars, cultures subjected to hypoxia in the presence of vehicle. Treatment with Allo did not prevent neuronal death induced by hypoxia (ANOVA for repeated measures Allo vs. vehicle for panel A: $F_{(1,8)}=4.4$, $p=0.10$; for panel B: $F_{(1,10)}=2.5$ $p=0.17$). Fischer PLSD post hoc test showed for both tissues Before and post vs. 24 h post: *$p<0.005$, data from 3 independent experiments.

contribute to increase oxidative stress and free reactive oxygen species and thus lead to cell death (Capani et al., 2001). Allo is a known potentiator of $GABA_A$ receptor function-mediated chloride influx (Crawley et al., 1986; Morrow et al., 1987), resulting in increased GABAergic inhibition. In this context, Allo could be beneficial by increasing inhibitory tonus and subsequently decrease excitotoxic cell death. Previous studies have shown that Allo and its precursor progesterone reduce excitotoxic cell death mediated through NMDA receptors and excessive release of postsynaptic glutamate (Ciriza et al., 2004; Lockhart et al., 2002).

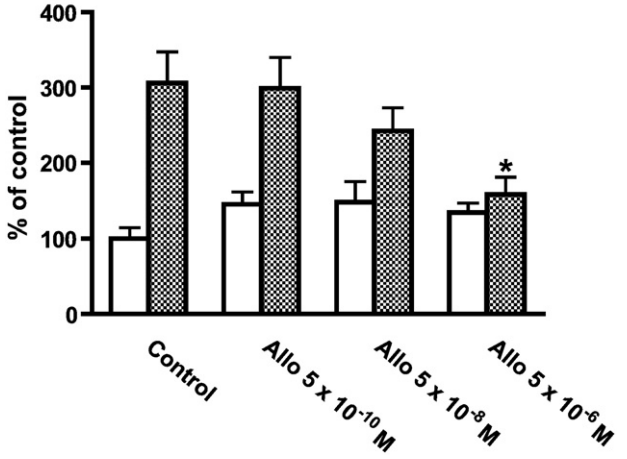

Fig. 3 – Effect of Allo on the astrogliosis induced by hypoxia in the neocortex. Empy bars, cultures treated with Allo or vehicle under normoxia. Filled bars, treated cultures subjected to 1 h of hypoxia. Two-way ANOVA showed a significant effect by hypoxia [$F_{(1,59)}=38.6$ $p<0.0001$] and by treatment [$F_{(3,59)}=2.8$ $p<0.05$]. Allo (5×10^{-6} M) treatment significantly decreased GFAP signal triggered by hypoxia in neocortical cultures (41±11%), *$p<0.05$, Fisher PLSD test.

On the other hand, steroids hormones have well-defined stimulatory and inhibitory effects on cell proliferation of different target cells by regulating cyclin genes and cyclin-dependent kinases (Sutherland et al., 1995). The precise mechanism involved in the regulation of astroglia proliferation by neurosteroids remains to be determined.

Reactive astrocytes produce neurotoxic substances and inflammatory products that kill vulnerable neurons in various brain pathologies (Mori et al., 2006). Moreover, gliotic tissue forms a physical barrier that prevents axonal

regeneration and neuritic growth (Tian et al., 2006), thus, we would have expected an increase on neuronal viability by decreasing astrogliosis. Allo was recently shown to enhance the production of CD55, a potent inhibitor of the inflammatory cascade, following contusion injuries of the cerebral cortex in rats (VanLandingham et al., 2007). Nevertheless, we did not find any increase in neuronal survival by Allo treatment. In accordance to our results, Allo did not increase embryonic neuronal viability following hypoxia in rat cerebral cortical cultures (Marx et al., 2000). In contrast, in hippocampal and cerebellar cultures, Allo increased neuronal survival after hypoxia (Ardeshiri et al., 2006; Frank and Sagratella, 2000). The differences in dose, length of treatment, and neuronal cell type may explain the discrepancy between these studies.

In contrast to the positive effect of Allo 5×10^{-6} M on gliosis, Allo 5×10^{-8} M and 5×10^{-10} M was observed to have a slight or nonsignificant decrease on the gliosis induced by hypoxia in both neocortical and hippocampal organotypic cultures. These could be directly related to the doses used. Moreover, it is possible that in vivo metabolisms of Allo to 5α-dihydroprogesterone or to its metabolite, 5α-pregnane-3α,20α-diol, both of them inactive on $GABA_A$ receptors (Purdy et al., 1990) decrease the ratio of the neuroactive steroid when lower concentrations were used.

On the other hand, we could not exclude a regulatory effect on neurogenesis. Recent data show that Allo is a potent neuroregenerative agent that promotes neuronal progenitor proliferation in the roden hippocampus and human cortex (Mellon, 2007; Wang and Brinton, 2008). In fact, a reduction of gliosis by neurosteroids has been associated with regeneration (Jones et al., 1997, 1999; Kujawa et al., 1991). Current studies in our laboratory are focused in determining whether Allo could stimulate neuronal regeneration upon hypoxia.

This study examines the neuroprotective effects of Allo upon asphyxia in organotypic cortical, striatal, and hippocampal neurons. It extends investigations demonstrating the importance of neurosteroids by providing evidence that neurosteroids also play a role in neuronal protection against hypoxia. Along pregnancy Allo concentration is high in fetal brain and increases further after an acute hypoxic stress (Hirst et al., 2006). The perturbation of the normal Allo levels in late gestation rends the fetal brain vulnerable to hypoxic-induced injury (Westcott et al., 2008; Yawno et al., 2007). If it is determined that Allo have neuroprotective effects following anoxic neurodevelopmental insults in vivo, then it could be used as a new pharmacologic treatment strategy for prenatal and perinatal anoxic injury. It is also worthy to consider the effect of Allo on gliotic tissue in the context of neurodegenerative changes associated to aging, since gonadal steroids levels show a profound decline in aging humans.

Previous studies in hippocampal organotypic culture have shown astrogliosis in response to NMDA exposure that parallels that seen in vivo (Kunkler and Kraig, 1997). In this context, both neocortical and hippocampal organotypical cultures reproduced the effect observed in our experimental model of perinatal asphyxia in vivo, i.e., a markedly increase of GFAP in response to hypoxia. Thus, the organotypic culture technique is a novel alternative model to study the mechanisms of hypoxic insult. Moreover, because endogenous neuroactive steroids are rapidly biotransformed when administered exogenously, this system offers the opportunity to explore direct effects of different drug treatments in the search of new therapeutic tools to reverse/attenuate the damage induced by hypoxia.

4. Experimental procedures

4.1. *Animals and organotypic cultures*

P3–P5 Sprague-Dawley rats were rendered unconsciously by CO_2 inhalation and immediately killed by decapitation. Sections (500 μm) from the premotor cortex containing the striatum and sections containing the hippocampus were obtained in oxygenated, sucrose-substituted solution (Seamans et al., 2001) using a Leica vibratome. Alternatively the hippocampus was dissected from brains. Three slices from each region or whole hippocampus were placed on a Millipore millicell insert in a six-well culture dish. One milliliter of serum-based media fed the slices from below. The plating media contained 50% basal medium Eagle, 25% Earle's balanced salt solution, 25% horse serum plus 6.5 mg/ml glucose, 25 mM HEPES–NaOH (pH 7.2), 100 μg/ml streptomycin and Glutamax for first 3 days. After 3–4 days inserts were placed in a fresh six-well dish with 1 ml of media as above but containing 70% basal medium and 5% horse serum instead.

4.2. *Allopregnanolone treatment and hypoxia*

After 6 days in vitro (DIV), media was replaced and cultures were exposed to Allo at three different concentrations, 5×10^{-10}, 5×10^{-8} or 5×10^{-6} M, or vehicle for 24 h at 37 °C/5% CO_2. The steroid was dissolved in 0.01% dimethyl sulfoxide (DMSO). Equal concentration of DMSO added in control wells has not influence on neuronal survival (data not shown). At 7 DIV after Allo or vehicle exposure the culture media was changed to fresh oxygenated media (normoxia group) or replaced by N_2 presaturated media (100% N_2, hypoxia group) and cultures were subjected to hypoxia for 1 h in an anaerobic chamber (Billups-Rothenberg, Inc.). Hypoxia was produced by a quick saturation of the chamber with a 100% N_2 atmosphere. Cultures media were then changed to fresh oxygenated media and cultures were placed at 37 °C/5% CO_2 for 24 h. For each experiment control slice cultures from the same animals and similar preparations were kept in other incubator under normoxic conditions. Experiments were repeated at least three times using tissues from three separate litters each time.

4.3. *LDH measurement*

Measurement of LDH activity in the extracellular medium was performed as a quantitative method for assessing cell injury by using a cytotoxicity kit assay (Cyto Tox 96 nonradiactive, Promega). The medium was taken at DIV 7 before and after the anoxic insult, and at DIV 8, and it was processed for LDH within the first 24 h. Data from five independent experiments were analyzed.

4.4. *Immunocytochemistry and confocal microscopy*

Sections were incubated in ice-cold 4% paraformaldehyde (PFH) in PBS pH 7.4 for 2 h, washed twice, and placed in PBS containing 0.1% Tween 20 and 10% normal goat serum (NGS) for 1 h at room temperature. The samples were then incubated with rabbit anti-GFAP (Sigma-Aldrich; 1:300) in PBS containing 2% NGS and 0.1% Tween 20 overnight at 4 °C. Subsequently, samples were rinsed in PBS for 30 min and then incubated with goat anti-rabbit 488 (Molecular Probes, 1:200) for 2 h at room temperature. Finally, after washing, samples were mounted on glass slides and examined with a Leica TCS SP inverted confocal scanning laser microscope. As control, primary antibody was omitted in some sections, and they were processed as described. In those control sections, the fluorescence staining intensity obtained was used as a marker to identify the positive staining since a low staining intensity similar to background was not regarded as positive immunoreactivity.

4.5. *Western blotting*

Sections collected at DIV 8 were rapidly frozen on dry ice and stored at −80 °C. Frozen tissue fragments were placed in 300 μl lyses buffer containing 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, and one minitablet protease inhibitor mixture (Roche Diagnostics) per 10 ml pH 7.4. Triton X-100 was added (1% final concentration), and the samples were incubated on ice for 30 min. Then samples were sonicated and centrifuged for 5 min at 5000×*g* at 4 °C. The supernatants were collected and protein concentrations were determined by Bradford (Bradford, 1976) using BSA as standard. Samples of supernatants containing 20 μg protein were heated to 100 °C for 2 min in 62 mM Tris–HCl buffer, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol and 10 mM 1,4-dithiothreitol. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in 8% gels in a Bio-Rad Mini-Protein II apparatus (Bio-Rad, Argentina).

4.6. *Statistical analysis*

Values are expressed as mean±SEM. At least three similar separate experiments were evaluated in all cases containing samples from two to three different animals/treatment. Results were evaluated using Student's *t*-test for two-group comparison. The specific values for steroid treatment comparisons were analyzed by analysis of variance (ANOVA) followed by Fisher Protected Least Significant Difference test. Differences were considered significant at $p < 0.05$. Results are reported in the corresponding figure legends.

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