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Research report

Role of allopregnanolone on cerebellar granule cells neurogenesis

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Abstract

The role of allopregnanolone on immature cerebellar granule cells (CGC) proliferation was studied. Allopregnanolone (0.1–1 μM) increased [^{3}H]thymidine incorporation and cell number determined by neuronal counting and by an MTT colorimetric assay. The effect of the neurosteroid was completely prevented by preincubation with 10 mM $MgCl_2$, 10 μM nifedipine, 10 μM picrotoxin or by 50 μM bicuculine. We conclude that ALLO affects cerebellar neurogenesis by increasing calcium influx through voltage-gated calcium channels and $GABA_A$ receptors activation.

Theme: Development and regeneration
Topic: Neurotransmitter systems and channels

Keywords: Allopregnanolone; Neurogenesis; Cerebellum; Calcium; Proliferation; $GABA_A$ receptor

Steroids act through a genomic mechanism of action [10]; however, certain metabolites of progesterone (allopregnanolone, 5α,3α reduced metabolite) and deoxycorticosterone (5α-tetrahydrodeoxycortecosterone) are potent modulators of $GABA_A$ receptors [1,2,18,25,30]. These compounds are called neuroactive steroids and they exert their effects through non-genomic mechanisms altering rapidly neuronal excitability [18,30]. Endocrine glands are a well established source of these neuroactive steroids, although it has also been demonstrated that the CNS is capable of synthesizing them de novo (neurosteroids) from cholesterol or intermediate precursors [1]. The presence of neurosteroid synthesizing enzymes was reported in mature neurons and glial cells [16,20,28]. The developing nervous system is also capable of synthesizing steroids [7,28]. Furthermore, the embryonic rat brain and the placenta are able to metabolize progesterone to allopregnanolone (5α-pregnan-3α-ol-20 one, ALLO) and the 3β reduced isomer, isopregnanolone (5α-pregnan-3β-ol-20 one, ISO) [24]. In the neonatal cerebellum of the rodent, a time when an active granule cell neurogenesis takes place, a transient increase in 3β-hydroxysteroid dehydrogenase activity promotes the synthesis of progesterone and its metabolites [29]. Despite all the available information, the physiological implications of the presence of neurosteroids during perinatal CNS development are still not well understood.

Neural activity triggered either by neurotransmitter-gated channels, $GABA_A$ and NMDA receptors, or voltage-gated calcium channels of the L-subtype ($VGCC_L$) play a regulatory role in the neurogenesis of the developing cortex and cerebellum [5,12,14,21].

The aim of this work was to study the role of ALLO, a potent positive modulator of $GABA_A$ receptors, on cerebellar granule cells (CGC) neurogenesis. We report that ALLO increased proliferation of immature CGC taken from 6- to 8-day-old rat pups. The effect was blocked by bicuculine ($GABA_A$ antagonist), picrotoxin (chloride chan-

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nel blocker) and nifedipine ($VGCC_L$). We postulate that ALLO increases DNA synthesis through a $GABA_A$ receptor activation and an increased calcium influx triggered by VGCC activation. These findings were partially reported previously in an abstract form [17].

1. Cell cultures

Animal care and manipulation was in agreement with institutional guidelines and the Guide for the Care and Use of Laboratory Animals [15]. Cell suspensions were obtained from cerebella of 6–8-day-old Sprague–Dawley rats and placed in Krebs–Ringer solution supplemented with 6 g/l glucose. After meninges were dissected out, the tissue was cut into 1-mm pieces and incubated in saline containing 0.025% trypsin (Sigma, St. Louis, MO), 1.2 mM $MgSO_4$ and 3 mg/ml bovine serum albumin (BSA, Sigma) for 15 min at 37 °C, with continuous agitation. Enzymatic digestion was stopped with ovomucoid (trypsin inhibitor, Sigma) and the tissue was mechanically dissociated with Pasteur pipettes of two different diameters (25 strokes), in saline containing ovomucoid and 0.01% DNAse (Roche, Indianapolis, IN). The cell suspension obtained was sedimented at 150*g* for 10 min and the pellet resuspended in Neurobasal media containing 5.4 mM KCl, supplemented with B27 (Neurobasal, Gibco, Grand Island, NY). Cells were seeded onto 96 multiwells (300,000 cells/well) or 24 multiwells (300,000 cells/well), coated with poly-D-lysine (MW 300,000, Sigma).

2. [^{3}H]Thymidine assay

Cells were seeded on 96 multiwells and 2 h later they were incubated with [^{3}H]thymidine (1 μCi/ml) for 22 h. In preliminary studied, cell densities between 150,000–300,000 were found to be the optimal for these experiments. The cells were then osmotically disrupted through a 2-min incubation with bidistilled water and harvested with a NUNC cell harvester coupled to glass fiber filters (Whatman, GF/C). Filters were washed seven times with bidistilled water and allowed to dry overnight. Radioactivity was counted in a liquid scintillation β-counter.

3. Cell counting

CGCs were seeded on 24 multiwells and allowed to grow for 48 h. Then, cells were counted in three fields of quadruplicate samples (1 mm^2) using an ocular with a grid, under phase-contrast optics at 400× magnification. Vital neurons were identified as phase-bright small rounded cells (6–7 μm diameter) with two or more processes, which, in preliminary experiments, revealed positive to the neuronal marker Tetanus Toxin Fragment C [5].

4. MTT assay

CGCs were seeded on 96 multiwells and incubated for 24 h. Preliminary studies revealed that a concentration of 300,000 cells/multiwell was optimal to obtain a linear relationship between absorbance and cell number. After the incubation period, 10 μl of 5-mg/ml 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma) were added to each well and cells were incubated for 4 h in a humidified atmosphere (37 °C, 5% CO_2). One hundred microliters of solubilization solution (10% SDS in 10 mM HCl) were added to each well and plates were allowed to stand overnight in the incubator (37 °C, 5% CO_2). Absorbance was measured using a scanning multiwell spectrophotometer (ELISA reader) at a wavelength of 570 nm.

5. Data

Data in Figs. 2 and 3 are expressed as the ratio: (experimental−control)/control. Controls for ALLO-treated cultures were samples treated with saline; controls in the group of ALLO+antagonist were samples treated with antagonist. As reported previously [5,6,12] in this report,

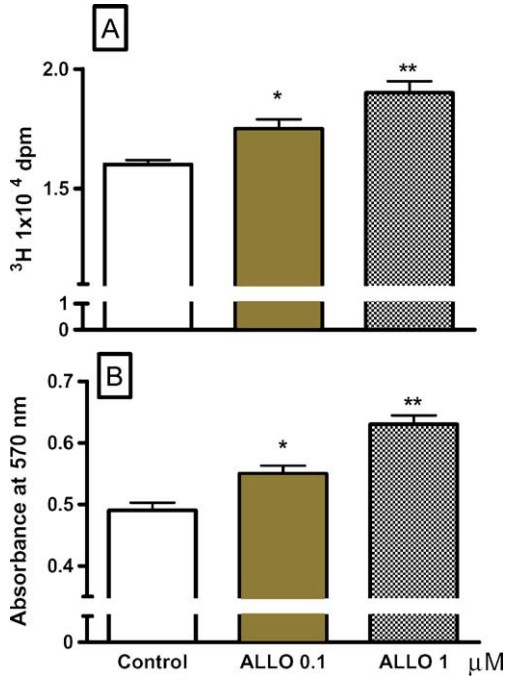

Fig. 1. Effect of 0.1 and 1 μM ALLO on [^{3}H]thymidine incorporation (A) and neuronal number measured by MTT assay (B). (A) [^{3}H]Thymidine was added to the cultures 2 h after seeding, incubated for 22 h and then cells were harvested. (B) Cultures were incubated with MTT for 4 h, then exposed to solubilization solution overnight; absorbance at 570 nm was measured. In all assays, ALLO or saline was added 2 h after seeding. Data are represented in A as dpm of [^{3}H]thymidine and in B as absorbance units at 570 nm, and represent mean±S.E.M. of 6–10 experiments. Statistical comparisons: $p<0.0001$ ANOVA. *$p<0.05$ saline vs. ALLO 0.1 and **$p<0.001$ saline vs. ALLO 1.

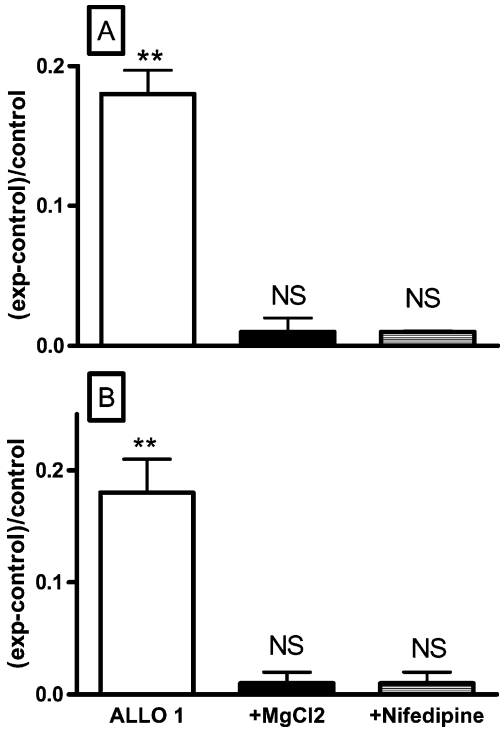

Fig. 2. Effect of calcium-channel blockers on ALLO-induced increase in proliferation. Preincubation with 10 mM $MgCl_2$ or 10 μM nifedipine prevented the increase in [^{3}H]thymidine incorporation (A) and the increase in absorbance at 570 nm (B) induced by ALLO (1 μM). Data are expressed as the ratio (experimental−control)/control. Controls are for ALLO, saline-treated samples and for ALLO+antagonist, samples with antagonist. Statistical comparisons: $p<0.0001$ ANOVA. **$p<0.001$ vs. saline; NS, $p>0.05$, vs. antagonist alone.

$MgCl_2$ and nifedipine did not change proliferation (in dpm, control: 15,904±231, n: 7; $MgCl_2$: 15,681±392, n: 5; nifedipine: 15,346±715, n: 5; $p>0.05$). In all cases, data are expressed as the mean±S.E.M. Statistical significance was examined by a one-way ANOVA with a Bonferroni post-test where $p<0.05$ was considered significant.

6. Drug treatment

In all assays, ALLO (0.1–1 μM) was added 2 h after seeding the cells. Nifedipine (10 μM), $MgCl_2$ (10 mM), picrotoxin (10 μM) and bicuculine (50 μM) were added 1 h after seeding the cells. All drugs were purchased from Sigma.

The effect of 0.1–1 μM ALLO on neuronal proliferation was studied in CGCs obtained from 6- to 8-day-old rats. During the first 24 h in vitro cells are rounded and able to incorporate [^{3}H]thymidine. ALLO was tested in a [^{3}H]thymidine incorporation assay (Fig. 1A). This neurosteroid induced a significant increase in [^{3}H]thymidine incorporation with both concentrations used ($p<0.0001$, ANOVA). Lower concentrations of ALLO (10 nM) failed to increase proliferation and a higher concentration (10 μM) was at the plateau level (in dpm: 1 μM ALLO: 19,441±495 (n: 11), 10 μM ALLO: 18,896±951 (n: 7), $p>0.05$). A significant increase in cell number was observed in a colorimetric assay (MTT assay, Fig. 1B, $p<0.0001$) and by cell counting (number of cells/mm^2: control: 474±18 (n: 9), ALLO 0.1 μM: 550±27 (n: 9), ALLO 1 μM: 643±27 (n: 9), $p<0.05$, ANOVA). To evaluate the role of Ca^{2+} as a possible second messenger mediating the neurosteroid-induced proliferation, we carried out experiments in the presence of nifedipine or $MgCl_2$. The increase in proliferation induced by ALLO, as evaluated by [^{3}H]thymidine incorporation (Fig. 2A) and MTT assays (Fig. 2B), was blocked by 10 mM $MgCl_2$ and 10 μM nifedipine. As reported previously, neither $MgCl_2$ nor nifedipine affected significantly (less than 2% from controls) cell proliferation and cell number (Fig. 2 and Refs. [5,12]).

To determine whether the effect of ALLO involved $GABA_A$ receptor participation, we performed a series of experiments in the presence of bicuculine, a $GABA_A$ receptor antagonist, and picrotoxin, a chloride channel blocker. As shown in Fig. 3A, the increase in [^{3}H]thymidine incorporation induced by ALLO was blocked by 10 μM picrotoxin and 50 μM bicuculine. The increase in cell number induced by ALLO, corroborated by the MTT assay, was blocked by 50 μM bicuculine and 10 μM picrotoxin (Fig. 3B).

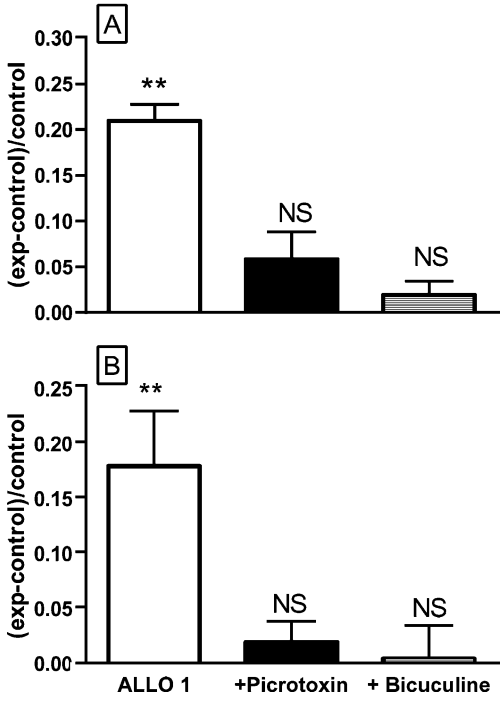

Fig. 3. Effect of a $GABA_A$ antagonist and a chloride channel blocker on 1 μM ALLO-induced increase in proliferation. Preincubation with 50 μM bicuculine and 10 μM picrotoxin prevented the increase in [^{3}H]thymidine (A) and MTT absorbance (B) induced by ALLO. Data are expressed as the ratio (experimental−control)/control. Controls are for ALLO, saline-treated samples and for ALLO+antagonist, samples with antagonist. Statistical comparisons: $p<0.0001$ ANOVA (A) and $p<0.01$ ANOVA (B). **$p<0.001$ vs. saline; NS, $p>0.05$, vs. antagonist alone.

Several reports demonstrate that neurosteroids play a neurotrophic role in the nervous system. During development neurosteroids stimulate dendritic growth in neocortical and Purkinje cells [8,26], synaptogenesis [26] and the survival of cultured brain neurons [4]. In adult rodents, they promote myelin formation and thus contribute to nerve regeneration [13,27]. We report herein that ALLO induces an increase in CGC proliferation suggesting that this natural agent may play a role in determining cell number in the developing CNS including humans, since ALLO effective concentrations in our model are similar to those reported in the last trimester of gestation [23]. The increased immature CGC proliferation induced by ALLO is mediated by the activation of $VGCC_L$ as it is blocked by nifedipine. In this regard, ALLO was able to increase Ca^{2+} in hypothalamic neurons; an effect also blocked by nifedipine and bicuculine [9], supporting a role for $GABA_A$ receptors in this mechanism. ALLO effect was blocked by $MgCl_2$, suggesting that an activation of NMDA receptors can take place. However, neither pretreatment with MK801 and CNQX, prevented the proliferative effect induced by VGCC activation [5] nor exogenously applied glutamate and NMDA increased proliferation in these experimental paradigm (Fiszman, unpublished observations). Therefore, Mg^{2+} may antagonize the effect of ALLO by blocking the accumulation of calcium.

The blockade of ALLO-induced proliferation by bicuculine and picrotoxin suggest that it maybe associated with $GABA_A$ receptor activation and chloride channel activity. Moreover, ALLO effective concentrations in our model are similar to those reported to modulate $GABA_A$ binding and chloride channel activity elicited by $GABA_A$ receptors [19,22]. In developing neurons, prior to maturation, $GABA_A$ receptors activation induces depolarizing responses, instead of hyperpolarizing ones that are mediated by a reversed chloride gradient [3]. GABA-mediated depolarization increases intracellular calcium concentration, which is a second messenger for the neurotrophic effects induced by GABA [11]. Furthermore, in this cell preparation, we have previously demonstrated that exposure to GABA induced an increase in CGCs proliferation [12] and a depolarizing response that in turn triggers calcium influx through the activation of $VGCC_L$ [6], both effects are dependent on $GABA_A$ receptor activation. Therefore, ALLO effects resemble those of GABA and can be accomplished by either modulating the endogenous GABA bound to the $GABA_A$ receptor, or by a direct action on the receptor. Since in our cell preparation GABAergic interneurons amount to only 5% of the total number of cells, it is possible that ALLO exerts its effect independently of the presence of GABA, and through a direct interaction with $GABA_A$ receptors [19]. However, an indirect effect can not be ruled out since a small concentration of GABA can be enough to activate the receptors.

In conclusion, our results indicate that neurosteroids induce proliferation of immature CGCs through a mechanism that involves extracellular calcium, activation of L-type VGCC and $GABA_A$ receptors.

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