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Progesterone and allopregnanolone exacerbate hypoxic-ischemic brain injury in immature rats

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ABSTRACT

Progesterone and its metabolite, allopregnanolone, are neurosteroids that are present at high concentrations in fetal brains that decrease right after birth. Allopregnanolone is a potent positive modulator of γ-aminobutyric acid A ($GABA_A$) receptor function. We examined the effect of exogenous administration of these steroids on hypoxic–ischemic encephalopathy in immature rats. Progesterone (10 mg/kg), allopregnanolone (10 mg/kg), or vehicle alone was intraperitoneally administered immediately before and then subcutaneously 6 h and 24 h after hypoxia–ischemia to postnatal day 7 (P7), day 14 (P14), and day 21 (P21) rats. The effects of the treatments were evaluated using histological analyses (hemispheric volumes and semi-quantitative scoring for neuropathologic injury). Both progesterone and allopregnanolone significantly exacerbated brain injury in P7 and P14 rats, but not in P21 rats. This detrimental effect was similar across the examined brain regions (the cortex, striatum, hippocampus, and thalamus) and showed no sex differences. Co-administration of the $GABA_A$ receptor antagonist, bicuculline, partially mitigated the exacerbating effect of allopregnanolone. Based on the similarity of the effects of these neurosteroids, we speculate that progesterone accentuates neuronal injury mainly via the activity of allopregnanolone. The present study indicates that the detrimental effects of allopregnanolone were, at least in part, mediated via GABAergic neuroexcitability. This is in line with the notion that GABA is excitatory for immature neurons, while it is inhibitory for mature neurons.

Introduction

Fetuses physiologically experience hypoxic conditions because they have a relatively low oxyhemoglobin saturation (65%) in their cerebral circulation (du Plessis, 2009). Hence, we assumed that fetuses might have innate mechanisms for coping with hypoxia and possibly protect themselves from hypoxia–ischemia (HI) better than children and adults. We hypothesized that certain compounds present at higher concentrations in the brain during the fetal period compared with other periods of life might have neuroprotective properties against hypoxia. Neonatal HI encephalopathy is caused by respiratory and/or circulatory insufficiency, and many survivors have long-term cognitive dysfunctions, as well as cerebral palsy (Lindstrom et al., 2006).

Progesterone (PROG) and its metabolite, allopregnanolone (ALLO, 3α-hydroxy-5α-pregnan-20-one, 3α,5α-tetrahydroprogesterone), are neuroactive steroid hormones that are also known as neurosteroids because they are synthesized *de novo* in the nervous system (Belelli and Lambert, 2005). PROG and ALLO are present at high concentrations in the brains of fetal rats and sheep (Grobin et al., 2003, Nguyen et al., 2003). These two neurosteroids are both supplied from the maternal circulation and synthesized in the fetal brain. Serum PROG and ALLO levels in pregnant women continue to increase during pregnancy, with the highest levels at term, i.e., 10 to 100 times higher than during preconception (Luisi et al., 2000). The levels of ALLO in umbilical cord blood are almost the same as those in maternal blood (Hill et al., 2000), and these steroids easily penetrate the brain (Wang et al., 2010). The PROG and ALLO concentrations in the fetal brain decrease right after birth, mainly due to the loss of the maternal blood supply (Grobin et al., 2003, Nguyen et al., 2003). Given that the fetal brain is exposed to high levels of PROG and ALLO, we hypothesized that these neurosteroids might have some neuroprotective properties against hypoxia and that an exogenous supply of these steroids might alleviate HI-induced brain injury in immature subjects. Erythropoietin, for example, which is prominent in the fetal brain, has shown to be neuroprotective in rodents with HI injury when administered exogenously after birth, and is

Abbreviations: HI, hypoxic–ischemic, hypoxia–ischemia; PROG, Progesterone; ALLO, allopregnanolone; P, postnatal day; $GABA_A$, γ-aminobutyric acid A; ANOVA, analysis of variance.

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currently being tested for infants with HIE and for extremely-low birth weight infants (Juul, 2000; McPherson and Juul, 2010).

Despite the appeal of the hypothesis that compounds present at high concentrations in the fetal brain could have neuroprotective properties, there is a critical concern regarding the use of PROG and ALLO in immature animals and humans. ALLO acts as a potent positive modulator of γ-aminobutyric acid A ($GABA_A$) receptors (Belelli and Lambert, 2005). GABA depolarizes immature neurons and is excitatory, while it hyperpolarizes mature neurons and is inhibitory (Ben-Ari et al., 2007). Therefore, PROG and ALLO treatment could potentially exacerbate neonatal HI encephalopathy through a neuroexcitatory mechanism involving $GABA_A$ receptors.

To our knowledge, no study has examined the effect of an exogenous supply of PROG or ALLO on immature animals with brain injury. The purpose of this study was to examine the effects of PROG and ALLO on immature rats with HI-induced brain injury.

Materials and methods

Hypoxia–ischemia

Seven-day-old (P7; experimental paradigm), 14-day-old (P14), and 21-day-old (P21) Wistar rat (Japan SLC, Hamamatsu, Japan) pups were prepared for surgery. All experiments were performed in accordance with protocols approved by the Experimental Animal Care and Use Committee of the National Cerebral and Cardiovascular Center. Rats were subjected to a modified Rice–Vannucci procedure to produce HI injury. The Rice–Vannucci model combines permanent ligation of the unilateral carotid artery with exposure to hypoxia for several hours in 7-day-old rat pups and has been widely used for numerous studies on the pathogenesis of HI injury (Rice et al., 1981; Johnston et al., 2005). The brain of newborn rats cannot be damaged by either anoxia alone or unilateral carotid artery ligation alone (Rice et al., 1981). Briefly, under isoflurane anesthesia (4.0% for induction and 1.5 to 2.0% for maintenance), the left carotid artery was permanently occluded. After a 1–2 h recovery period, the P7, P14, and P21 rats were subjected to hypoxia (8% oxygen and 92% nitrogen, at 33.0 °C) for 120, 80, and 50 min, respectively. The duration of the hypoxic exposure was optimized to obtain a similar degree of brain injury in each group as assessed by hemispheric volume and neuropathological scores. After 1 h recovery in a temperature-controlled incubator, rats were returned to the dams until sacrifice.

Drug administration

PROG (Sigma-Aldrich, St. Louis, MO) and ALLO (Calbiochem/EMD Biosciences, San Diego, CA) were dissolved in 22.5% (2-hydroxypropyl)-β-cyclodextrin. Bicuculline (Sigma-Aldrich, St. Louis, MO) was dissolved in hydrochloric acid and then titrated to pH 5.2 by adding sodium hydroxide and phosphate-buffered saline (PBS). A total of five different experimental groups were used: regular dose paradigm in P7, P14, and P21 rats, low dose paradigm in P7 rats, and bicuculline paradigm in P7 rats. Ten to fourteen littermates, both males and females, were randomly assigned to one of three or four different treatment groups. As sex differences were designed to be assessed in Experiment 1 (P7), double the number of littermates was assigned to each treatment group, so that each sex group consisted of approximately 10 pups. As four different treatment groups were assessed in Experiment 3 (P7), 14–20 pups were used.

Experiment 1 (P7): To produce physiological prenatal levels of the two steroids in P7 rats (the level of brain maturation in P7 rats is generally considered comparable to that of P0 human neonates (Dobbing and Sands, 1979), although other authors have suggested that P12–13 rats fulfill this criterion (Romijn et al., 1991) (Clancy et al., 2007)), PROG and ALLO were each administered at a dose of 10 mg/kg body weight (5 mg/ml) immediately before the start of the hypoxic exposure. To simulate the clinical situation of treating newborn babies in the P7 rats, the steroids were administered 6 and 24 h after the start of the hypoxic exposure. The first injections (immediately before hypoxia) were given intraperitoneally to ensure rapid absorption, and the subsequent injections were given subcutaneously for more gradual absorption. The vehicle was administered in the same manner. This protocol is based on the one reported for neuroprotective effects in adult rats with stroke (Jiang et al., 1996, Sayeed et al., 2006), with minor modifications.

Experiment 2 (P7): In this protocol, ALLO was administered at a dose of either 3 mg/kg or 1 mg/kg. Other than the dosage, the protocol was same as that used in experiment 1. The vehicle was also administered in the same manner.

Experiment 3 (P7): Littermates were randomly assigned to one of four groups: vehicle (PBS) + vehicle (β-cyclodextrin), bicuculline + vehicle (β-cyclodextrin), vehicle (PBS) + ALLO, or bicuculline + ALLO. ALLO and the vehicle (β-cyclodextrin) were both administered in the same manner as that described in experiment 1. The $GABA_A$ receptor antagonist, bicuculline (2 mg/kg), and its vehicle (PBS adjusted to pH 5.2) were each administered intraperitoneally just before and subcutaneously 6 h after each ALLO injection, for a total of 5 injections (Fig. 1A). This protocol is based on one used previously to study the effects of $GABA_A$ blockade in immature rats (Galanopoulou, 2008).

Experiments 4 (P14) and 5 (P21): The same protocol used in experiment 1 was used for P14 and P21 rats.

Quantitative histological analysis

Seven days after the HI insult, the rats were deeply anesthetized with an overdose of pentobarbital and perfused with saline followed by 4% formaldehyde via the left ventricle. After perfusion, the brains were removed and sectioned coronally into 2-mm slices using a rat brain slicer (Neuroscience Inc., Tokyo, Japan). The area (mm^2) of the contralateral and ipsilateral hemispheres in each brain section was measured using NIH Image software (ImageJ, 1.43r). The hemispheric volume of each brain was estimated by summing the hemispheric area of the brain slices and multiplying by the section interval thickness. The injury was evaluated in hematoxylin-eosin-stained sections from four brain regions (cortex, striatum, hippocampus, and thalamus). The system we previously developed for evaluating neuropathologic injury (Tsuji et al., 2004) was used in the present study. Neuropathologic injury in the cerebral cortex was scored from 0 to 4 (0: no injury, 4: extensive confluent infarction). Neuropathologic injury in the hippocampus, striatum, and thalamus was scored from 0 to 6. The total score (0–22) was the sum of these ratings. Both hemispheric volume measurement and neuropathological scoring were assessed blindly.

Statistics

The effects of the neurosteroid treatment on the cerebral hemispheric volumes were assessed using a two-way analysis of variance (ANOVA) followed by Bonferroni's test. The injury scores were not distributed normally, so differences in injury scores were assessed using a Kruskal–Wallis test, followed by Dunn's multiple comparison. Sex differences in the injury scores were assessed using Mann–Whitney *U* test with Bonferroni's correction for multiple comparisons. The death rate of the animals was analyzed using Fisher's exact test with Bonferroni's correction for multiple comparisons. The differences in body weight and in rectal temperature were analyzed using a one-way ANOVA, followed by Bonferroni's test. Differences were considered significant at $P<0.05$. The results are presented as the mean ± standard error of the mean (SEM).