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Sex difference in sensitivity to allopregnanolone neuroprotection in mice correlates with effect on spontaneous inhibitory post synaptic currents

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ABSTRACT

Allopregnanolone (ALLO) is a neurosteroid that has many functions in the brain, most notably neuroprotection and modulation of gamma-amino butyric acid (GABA) neurotransmission. Using a mouse model of cardiac arrest and cardiopulmonary resuscitation, we have previously demonstrated that ALLO protects cerebellar Purkinje cells (PCs) from ischemia in a $GABA_A$ receptor-dependent manner. In this study we examined the effect of sex on ALLO neuroprotection, observing that low dose ALLO (2 mg/kg) provided greater neuroprotection in females compared to males. At a higher dose of ALLO (8 mg/kg), both sexes were significantly protected from ischemic damage. Using an acute cerebellar slice preparation, whole cell voltage clamp recordings were made from PCs. Spontaneous inhibitory post synaptic currents (IPSCs) were analyzed and the response to physiological ALLO (10 nM) was significantly greater in female PCs compared to male. In contrast, recordings of miniature IPSCs, did not exhibit a sex difference in response to ALLO, suggesting that ALLO affects males and females differentially through a mechanism other than binding postsynaptic $GABA_A$ receptors. We conclude that the female brain has greater sensitivity to ALLO mediated potentiation of GABAergic neurotransmission, contributing to increased neuroprotection.

1. Introduction

Cardiac arrest requiring cardiopulmonary resuscitation (CA/CPR) is a leading cause of death and disability in the United States, affecting over 300,000 adults and children in the United States each year (Roger et al., 2010). Gender is a significant factor influencing incidence and possibly outcome, with women being relatively protected compared to men (Kim et al., 2001; Rosamond et al., 2008; Vukmir, 2003; Wigginton et al., 2002). Animal models of cerebral ischemia, including CA/CPR, mimic the human epidemiology, exhibiting sex specific differences in tissue damage and long-term behavioral recovery (For Review See Herson and Hurn, 2010). Innate protection from ischemic damage in the female is due in large part to endogenous levels of sex steroids, the estrogens and progesterone. In addition to the estrogens and progesterone, there is emerging evidence that metabolites of these hormones have important physiological and pathophysiological roles in the brain (For review see Liu et al., 2010).

The neurosteroid allopregnanolone (ALLO) is a metabolite of progesterone that is neuroprotective in several animal models of neurodegenerative diseases including Alzheimer's (Brinton and Wang, 2006), traumatic brain injury (TBI) (Djebaili et al., 2004), stroke (Sayeed et al., 2006), and recently cardiac arrest (Kelley et al., 2008). Cerebral ischemia, as experienced following CA/CPR results in selective damage to vulnerable neuronal populations, including hippocampal CA1 neurons (Pulsinelli et al., 1982) and cerebellar Purkinje cells (Ardeshiri et al., 2006; Brasko et al., 1995; Fonnum and Lock, 2000; Horn and Schlote, 1992). We have previously demonstrated that ALLO can protect cultured PCs from *in vitro* ischemia, oxygen–glucose deprivation, and global ischemia *in vivo*, in part by potentiating $GABA_A$ receptor activity (Ardeshiri et al., 2006) and also by preserving $GABA_A$ receptor protein and function (Kelley et al., 2008). To date, the interaction between animal sex and ALLO has not been examined in the context of neuroprotection. In the current study, we tested whether there is a sex difference in ALLO neuroprotection of PCs and we examined the ability of ALLO to enhance GABAergic neurotransmission in the cerebellum.

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2. Methods

2.1. Cerebellar slice preparation

All animal experiments were performed in accordance with the National Institutes of Health guidelines for care and use of Laboratory animals and approved by the Oregon Health and Science University Animal Care and Use Committee. Adult (8–16 wk old) C57/Bl6 male and female mice (Charles River) were anesthetized by i.p. injection of a ketmine/xylazine (80/12 mg/ml, Sigma Aldrich) cocktail and transcardially perfused with ice cold oxygenated cutting solution (see 2.3). Mice were decapitated, cerebellum removed, and sagittal slices (400 μm thick) were cut from the vermis of the cerebellum with a vibroslicer (Vibratome or Leica). Slices were incubated at 37 °C in warm oxygenated artificial cerebrospinal fluid (ACSF, see 2.3) for 30 min then stored at room temperature. All experiments were performed at room temperature and completed within 4–5 h of slicing to ensure cell viability.

2.2. Electrophysiology

Whole cell voltage clamp recordings were made from the soma of PCs using an Axopatch 200B (Axon Instruments, Union City, CA) amplifier interfaced to a Dell computer (Dell, Round Rock, TX). Data was collected using pCLAMP9 (Molecular Devices, Sunnyvale, CA) at a sample frequency of 20 kHz, with lowpass filtering at 2 kHz. Electrodes pulled from borosilicate glass capillaries with inner filaments using a Flaming Brown electrode puller (Sutter Instrument Co, Novato, CA) had resistances of 2–4 MΩ when filled with a CsCl internal pipette solution (see 2.3). Whole cell capacitance and resistance were electronically compensated. Adequate whole cell access ($R_a < 30$ MΩ) was verified at the beginning of the recording, before recording in the presence of ALLO, and at the end of recording. Slices were continuously perfused with aerated ACSF using a gravity fed perfusion system with a flow rate of 1–2 ml/min.

Purkinje cells were voltage-clamped at a membrane potential of −60 mV and GABA-mediated spontaneous inhibitory post synaptic currents (sIPSCs) were recorded as inward currents (Herson et al., 2003; Konnerth et al., 1990). IPSCs were recorded in 3 min sweeps. TTX (250 nM) was bath applied for 6 min prior to recording baseline miniature IPSCs (mIPSCs) and TTX remained present during ALLO application. ALLO (10 nM) was bath applied for 20 min prior to recording its effect on IPSCs. Individual cells were used separately to test the effect of ALLO on sIPSCs or mIPSCs. Recordings were analyzed using Clampfit (Axon Instruments), and Igor Pro software (WaveMetrics, Lake Oswego, OR). Analysis of IPSC amplitude, frequency and kinetics were analyzed as described previously (Herson et al., 2003). Briefly, kinetic analysis was performed by detecting individual IPSCs using a sliding variable amplitude template. 20–30 events from each cell per condition were chosen at random, event baseline was adjusted by subtracting the mean baseline of the events, events were aligned by rise time, and event traces were averaged (Clampfit). Using a sum of exponentials function, decay constants were calculated (Igor). Event detection and fitting were confirmed by eye. The total number of events observed in 3 min recordings from each cell was used to calculate frequency (Igor). The amplitude of all events detected for each cell during a 3 min recording was used to calculate average IPSC amplitude and cumulative probability of amplitude for that cell, and data are reported as mean ± SEM. Unless otherwise noted, *n* represents the number of recordings of individual cells from separate animals. Additionally, recordings were only made from slices that were not previously exposed to exogenous ALLO.

2.3. Solutions and drugs

Cutting solution was composed of (in mM) 110 choline chloride, 2.5 KCl, 7 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 Dextrose, 11.6 Na-ascorbate, 3.1 Na-pyruvate. The composition of the ACSF solution was (in mM): 119 NaCl, 2.5 KCl, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 1.3 $MgCl_2$, 2.5 $CaCl_2$, and 10 Dextrose, aerated with 95% O_2/5% CO_2. Internal pipette solution was (in mM): 140 CsCl, 1 EGTA, 10 HEPES, 1 $MgCl_2$, 5 MgATP, pH of 7.3 with CsOH. TTX was dissolved in DMSO to make a stock concentration of 1 mM, working concentration was 250 nM. For electrophysiology recordings, ALLO was dissolved in DMSO at a stock concentration of 10 mM for storage. Fresh working solution of ALLO (10 nM) was made by serial dilution in ACSF daily. ALLO for *in vivo* CA/CPR experiments was dissolved in 20% β-cyclodextran in 0.9% saline. ALLO was obtained from Calbiochem/EMD Chemicals (Gibbstown, NJ). TTX was obtained from Tocris (Ellisville, MO).

2.4. Cardiac arrest & cardiopulmonary resuscitation (CA/CPR)

CA/CPR was performed on adult (20–25 g) male and female C57BL/6 mice to simulate global cerebral ischemia as described by our group previously (Kelley et al., 2008; Kofler et al., 2004). Anesthesia was induced with 3% isoflurane and maintained with 1.5–2% isoflurane in O_2 enriched air via face mask. Temperature probes were inserted into left temporalis muscle and rectum to monitor head and body temperature simultaneously. For drug administration, a PE-10 catheter was inserted into the right internal jugular vein and flushed with heparinized 0.9% saline. Animals were then endotracheally intubated, connected to a mouse ventilator (Minivent, Hugo Sachs Elektronik, March-Hugstetten, Germany) and set to a respiratory rate of 160 min^{-1}. Tidal volume was adjusted according to body weight to maintain arterial CO_2 tension within physiological range (35–45 mm Hg). CA was induced by injection of 50 μl KCl (0.5 M, 4 °C) via the jugular catheter, and confirmed by EKG. The endotracheal tube was disconnected from the ventilator and anesthesia stopped. During CA, body temperature was cooled to assure ease of resuscitation by placing the mouse on an ice water-filled pad. However, mouse head temperature was maintained at 38.5 °C, to assure reproducible injury, by a heated water-filled coil. CPR began 10 min after induction of CA by injecting 0.5 ml warm epinephrine solution (16 μg/ml), chest compressions at a rate of 300 min^{-1}, ventilation with 100% O_2 at a rate of 190 min^{-1}, and 25% increased tidal volume. At initiation of CPR, head temperature was cooled to 37 °C and the body re-warmed using a heat lamp and pad. Cardiac compressions were stopped when spontaneous circulation was restored. Catheters and temperature probes were removed and skin wounds closed. CPR was stopped and animal excluded from experiment if circulation was not restored within 2.5 min. CA survivors received post-operative care and survived 48 h post CA/CPR. ALLO was administered by ip injection (either 2 mg/kg or 8 mg/kg) 30 min prior to CA/CPR, with two subsequent boosting injections (at 6 h & 24 h), as described previously (Kelley et al., 2008). Vehicle (see 2.3) was administered at the same time points used for ALLO.

2.5. Histology

Following CA/CPR and 48 h recovery, mice were euthanized with isoflurane, perfused through the left ventricle with saline, followed by cold 4% formaldehyde, then brains were removed and post-fixed in formaldehyde for an additional 12 h. After removal, brains were coded with randomly assigned numbers. Brains were dehydrated, cleared, and embedded in paraffin. Coronal sections were made through the cerebellum at approximately −5.8 mm from Bregma at a thickness of 6 μm. FluoroJade B staining was used to label injured neurons (Schmued and Hopkins, 2000) as previously described by Kelley et al., 2008. Experimenter performing analysis was blinded to treatment. PC damage was expressed as percentage of FluoroJade positive PCs in the vermis of each cerebellum. *N* represents the number of mice examined under each condition. For each *n*, all PCs in a single 6 μM section (approximately 300 cells) were analyzed to determine the percentage of FluoroJade positive PCs.

2.6. Statistical analysis

All data is presented as mean ± SEM. Each *n* represents an individual cell for electrophysiology experiments and an individual animal for *in vivo* experiments. Statistical significance was determined using students *t*-test (unpaired, 2-tailed, if $P < 0.05$) or two way analysis of variance (ANOVA) with Neuman–Keuls post hoc analysis, $P < 0.05$ to assess sex versus drug interaction.

3. Results

3.1. ALLO neuroprotection following CA/CPR

We have previously demonstrated that ALLO can protect PCs from global ischemia in male mice at a dose of 8 mg/kg (Kelley et al., 2008). However, ALLO's neuroprotective effects have not been examined in female mice. Using our mouse CA/CPR model we tested whether ALLO is neuroprotective in females and if the dose dependence is similar to males. Two doses of ALLO were tested, either 2 mg/kg or 8 mg/kg, administered by ip injection 30 min prior to CA/CPR followed by two boosting injections of the same dose at 6 h & 24 h recovery. We found that both males and females were significantly protected with the 8 mg/kg dose of ALLO. The percentage of FluoroJade positive PCs for males decreased from 20.09 ± 3.94% with vehicle treatment ($n = 11$) to 9.87 ± 2.06% with 8 mg/kg ALLO ($n = 13$, Fig. 1). The percentage of FluoroJade positive PCs for females decreased from 13.25 ± 2.39% with vehicle ($n = 6$) to 5.64 ± 2.18% with 8 mg/kg ALLO ($n = 6$, Fig. 1). With a 2 mg/kg dose of ALLO males showed almost no protection (19.00 ± 5.12%, $n = 8$), while females were strongly protected (5.43 ± 2.84% $n = 7$; $P = 0.06$ when compared to female vehicle; Fig. 1). Post hoc analysis revealed that response to 2 mg/kg was significantly affected by sex ($P > 0.05$, 2-way ANOVA). While not significant, our data indicates that vehicle treated females suffered less PC damage compared to males, consistent with female neuroprotection attributed to