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Perinatal intermittent hypoxia alters γ-aminobutyric acid: a receptor levels in rat cerebellum

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ABSTRACT

Perinatal hypoxia commonly causes brain injury in infants, but the time course and mechanisms underlying the preferential male injury are unclear. Intermittent hypoxia disturbs cerebellar γ-aminobutyric (GABA)-A receptor profiles during the perinatal period, possibly responding to transient excitatory processes associated with $GABA_A$ receptors. We examined whether hypoxic insults were particularly damaging to the male rodent cerebellum during a specific developmental time window. We evaluated cerebellar injury and $GABA_A$ receptor profiles following 5-h intermittent hypoxia (IH: 20.8% and 10.3% ambient oxygen, switched every 240 s) or room-air control in groups of male and female rat pups on postnatal d 1–2, wk 1, or wk 3. The cerebella were harvested and compared between groups. The mRNA levels of $GABA_A$ receptors α6, normalized to a house-keeping gene GAPDH, and assessed using real-time reverse-transcriptase PCR assays were up-regulated by IH at wk 1, more extensively in male rats, with sex influencing the regulatory time-course. In contrast, $GABA_A$ α6 receptor protein expression levels, assessed using Western blot assays, reached a nadir at wk 1 in both male and female rats, possibly indicating involvement of a post-transcriptional mechanism. The extent of cerebellar damage and level of apoptosis, assessed by DNA fragmentation, were greatest in the wk 3 IH-exposed group. The findings suggest partial protection for female rats against early hypoxic insult in the cerebellum, and that down-regulation of $GABA_A$ receptors, rather than direct neural injury assessed by DNA fragmentation may modify cerebellar function, with potential later motor and other deficits.

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1. Introduction

Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter in the nervous systems of widely divergent species. However, GABA is principally excitatory during the first postnatal week in rats, prior to the maturation of the glutamatergic system (Ben-Ari, 2002; Tyzio et al., 2007). As opposed to the adult, activation of $GABA_A$ receptors during this early period induces membrane depolarization and opens voltage-sensitive calcium channels (Leinekugel et al., 1999), which can be neurotoxic to immature neurons (Kaindl et al., 2008; Kim et al., 2008a; Olney et al., 2002).

Cerebellar outflow processes, consisting of GABAergic neurons, affect a range of somatomotor, autonomic motor (Lutherer et al., 1983; Zhu et al., 2006) and cognitive functions (Schmahmann et al., 2007; Tavano et al., 2007), and serve chemosensing (Xu and Frazier, 1997) and coordination of breathing (Monin et al., 1999) roles. Extensive evidence indicates that adult cerebellar neurons, including Purkinje cells, are predominantly GABAergic (Takayama, 2005) and are inhibitory in function. The developmental period in which the GABA system transitions from excitatory to inhibitory neurotransmission may make neural structures dependent on GABA function vulnerable to respiratory insults.

Of all sources of injury in the perinatal period, hypoxic exposure poses a primary risk, and is particularly a concern in the neonatal period, especially in premature infants, where the incidence of periodic breathing, obstructive events, or central apnea is high (Hibbs et al., 2008; Dayyat et al., 2007). Cerebellar injury resulting from hypoxic exposure has the potential to impact a range of physiological functions concurrently at the time of injury, and, because of extensive forebrain projections to cognitive and motor integrative sites, later in life (Biran et al., 2011; Taylor et al., 2006). Cerebellar pathology can contribute to impaired coordination of motor

Abbreviations: $GABA_A$, γ-aminobutyric acid (GABA)-A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IH, intermittent hypoxia; LSD, least significant difference; RT-PCR, reverse-transcriptase polymerase chain reaction; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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behavior, a principal cerebellar function. A corollary of that loss of motor coordination may be the potential for impaired breathing control, since respiration incorporates coordination of numerous thoracic, abdominal and upper airway muscles. Respiratory impairments are routinely observed in developmental pathological breathing patterns resulting from cerebellar injury, such as Arnold-Chairi malformations in infants, as well as adult breathing disorders found after cerebellar surgery or tumors (Chen et al., 2005; Chokroverty et al., 1984). Determining processes underlying early cerebellar injury has the potential to reveal mechanisms contributing to multiple motor, cognitive and breathing pathologies.

Exposure to conditions resulting in brain injury frequently shows relative protection in females (Berry et al., 2009). Steroid hormones modulate GABA cellular responses to differentiate male and female brains during the perinatal period (Schwarz and McCarthy, 2008). Profound sex differences appear in specific nuclei of the mid-brain (Sawada and Shimohama, 2000), and in the frequencies and types of synapses in estradiol-concentrating brain regions. The hormonal effects may serve to alter sensitivity of particular brain sites to hypoxia or other injury (Nuñez and McCarthy, 2008). However, the developmental time course of vulnerability to injury from hypoxia in relation to sex remains unclear. Animal models of intermittent hypoxic exposure suggestive of obstructive sleep apnea in humans show multiple structural and functional changes in the brain, with detrimental injuries to the hippocampus, frontal, and cerebellar cortex (LaManna et al., 2004; Gozal et al., 2010; Pae et al., 2005).

We aimed to analyze postnatal $GABA_A$ receptor α1, α3, and α6 subunit expression in the GABA-rich-cerebellum at different developmental stages in response to intermittent hypoxia exposure. We proposed that the neurotransmitter action resulting from expression of $GABA_A$ receptors in the cerebellum subjected to short-term intermittent hypoxia (IH) is age- and sex-dependent, and that this GABA-mediated action would result in age- and sex-related injury to $GABA_A$ receptor containing neurons in the cerebellum. To examine these propositions, we tested the following hypotheses: first, $GABA_A$ receptors expressions are disrupted by perinatal hypoxic challenges at a specific temporal window during an early developmental stage. Second, the degree of disruption is more prominent in males.

2. Experimental procedures

2.1. Animal preparation and tissue harvest

Eight near-end-term pregnant Sprague-Dawley rats (5–6 wk gestation) were housed in separate cages and maintained until parturition. After birth, two dams and their respective pups (usually 12–13 pups) were kept in two separate cages and designated the control group. This control group was housed in a commercially designed chamber (30 in. × 20 in. × 20 in.) that was operated under 12-h light–dark cycle (light cycle: 0600–1800) for 5 h. Two additional dams with their pups were designated Experimental Group Day 1 (or d 1) and were housed under room air (20.8% O_2), alternating with 10.3% O_2, balance N_2, every 240 s for 5 h at postnatal d 1–2 (between 24 h and 36 h after birth). Two other dams with their pups exposed to IH at postnatal 1 wk were designated Experimental Group wk 1. The remaining two dams and pups, exposed to IH for 5 h at postnatal 3 wk, were designated Experimental Group wk 3. After exposure to intermittent normoxic and hypoxic conditions for 5 h, all rats were maintained in separate cages under ambient air conditions until euthanasia at the age of 3 wk. Thus, pups in the Experimental Group wk 3 were sacrificed immediately after completion of IH exposure, and pups in the Group d 1 were sacrificed after 3 wk of recovery from the IH insult. Ten pups of each sex per group (d 1, wk 1, wk 3 and control) were randomly selected; thus, 80 pups constituted the study material in total.

Oxygen concentrations of the chamber were continuously monitored by an O_2 analyzer (ProOx 110, BioSpherix Instruments, Redfield, NJ), which adjusted moment-to-moment desired O_2 concentrations automatically. Alterations to the designated O_2 concentrations were induced by addition of N_2. Ambient CO_2 levels in the chamber were maintained at 0–0.1% by an infra-red analyzer (Capstar-100 CWE Inc., Ardmore, PA). Temperature and humidity levels were maintained at the same level as room air (24 °C and 68%).

Of 80 neonatal rats, 40 were male and 40 were female; 2 male and 2 female pups in each group, (16 pups total), were used to assess DNA integrity. Five male and 5 female pups per group, (40 pups total), were used for protein assays, and 3 male and 3 female pups per group, (24 pups total), were used for mRNA analyses. All rats were euthanized at 3 wk of age. After gender confirmation, the animals were anesthetized with pentobarbital (100 mg/kg, *i.p.*) and perfused with 100 mL of 0.1 M phosphate-buffered saline three times. The brains were rapidly harvested, and immediately stored in liquid nitrogen (−80 °C) for protein and mRNA analyses. The animal care and experimental protocols were approved by the local Animal Review Committee (ARC #2005-093-03) and complied with the National Institutes of Health guide for the care and use of laboratory animals.

2.2. Real-time RT-PCR

Total RNA was extracted from frozen cerebella using RNeasy mini kits (Qiagen, Valencia, CA; Cat #74106) and quantified using a Nanodrop spectrophotometer (ND-3300, Wilmington, DE) after RNA quality was assessed by the ratio between ribosomal RNAs (28s:18s) peaks using an automated electrophoresis system Agilent 2100 bioanalyzer (Agilent Technologies, USA) in conjunction with RNA LabChip kits.

Forward- and reverse-primers designed using the Beacon Designer 5.10 software (Premier Biosoft International, Palo Alto, CA) for $GABA_A$ receptors were the following:

$GABA_A$ receptor α1 (Forward: TGTTCTCAACGCAGTGATTCC; Reverse: CTCCTCCTCTTGTCTGTCTCC, amplicon size, 205 bp), $GABA_A$ receptor α3 (Forward: TTCTCCACCATCTCCAAGG; Reverse: TGCTGCCACTATTATCTACTG, amplicon size 261 bp), and $GABA_A$ receptor α6 (Forward: AGTCCCAGAAAGCCGAAAGG; Reverse: CCTCAGAAGATGGAACGATTGG, amplicon size, 168 bp).

GAPDH (Forward: TCATGAAGTGTGACGTTGACATCCGT; Reverse: CCTAGAAGCATTTGCGGTGCAGGATG, amplicon size, 285 bp).

Construction of the primers was outsourced (Integrated DNA technologies, Inc.) and PCR products from a cerebellum were sequenced and visualized using Sequence Scanner program (Applied Biosystem, vol. 1). Highly pure parts of the sequence, as defined by the software, were compared with published sequences of the genes using the Blast-2-sequence web-based program for validation of the PCR products.

The cDNA was quantified for $GABA_A$ α1, $GABA_A$ α3, $GABA_A$ α6, and GAPDH gene expression using the primer pairs. Real-time reverse-transcriptase polymerase chain reaction (RT-PCR) gene amplification, as determined by intercalated SYBR-Green total fluorescence emission (iQ SYBR Green Master Mix, Bio-Rad, Hercules, CA), was measured using the iCycler System (Bio-Rad, Hercules, CA) with the following cycle parameters: 1 cycle of 95 °C for 3 min followed by 45 cycles of 95 °C for 30 s, 55 °C for 20 s, and 72 °C for 30 s. Data analysis was performed using the iCycler iQ Optical System software (Bio-Rad, Hercules, CA). The PCR sample reactions were repeated in triplicate (Table 1). Relative gene expression levels normalized to a classic reference gene, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were calculated using the comparative C_T ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen, 2001). The iCycler iQ Optical System software was used to assess the quality of the mRNA. Correlation coefficients were 0.997 for GAPDH, 0.998 for $GABA_A$ α1, 0.995 for $GABA_A$ α3, and 0.997 for $GABA_A$ α6. PCR efficiency was 100.2% for GAPDH, 94.5% for $GABA_A$α1, 84.5% for $GABA_A$ α3, and 97.2% for $GABA_A$ α6. Melt curves consistently showed peaks well-superimposed.

2.3. Protein extraction and Western blot assay

One tablet of protease inhibitor (Roche Applied Science; Cat #1836170) was dissolved in RIPA buffer (Pierce, #89900: 25 mL Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Harvested cerebellar tissues, pooled from five brains per group, were added to ten volumes of RIPA buffer with protease inhibitor and homogenized using Polytron (# PT 10–35, Bohemia, New York). Working samples were incubated on ice for 30 min, centrifuged, and the supernatant was collected; a Bio-Rad DC protein assay using bovine serum albumin standard was performed to determine protein concentration for Western blot assays with respect to a standard curve.

Eighty μg of total proteins were sampled at each well and separated on 4–12% Tris–Glycine Gel (Invitrogen; Cat #EC60352). The protein bands were transferred polyvinylindene difluoride (PVDF) membranes that were blocked with 5% non-fat dry milk in 1× PBS buffer for 1 h at room temperature. Subsequently, the membranes were incubated with primary anti-$GABA_A$ receptor α1 (1:500; Upstate, Temecula, CA; Cat #06–868), anti-$GABA_A$ receptor α3 (1:200; Sigma, Saint Louis, MO; Cat #G4291) or anti-$GABA_A$ receptor α6 (1:1000; Chemicon International, Temecula, CA; Cat #AB5610), and followed by secondary horseradish peroxide-conjugated goat-anti-rabbit antibodies (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA). After incubation in SuperSignal West Pico solution (Pierce) for 10 min, the membranes were visualized in Bio-Rad ChemiDoc (Bio-Rad, Hercules, CA). For a reference protein, the membranes were probed with 1:3000 dilution of primary β-Actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA; Cat #SC-1616-R), followed by the same secondary antibody. Gels were scanned, and density of each lane was measured by a densitometer to convert them into graphs. Gel density of the reference protein was quantified, and then factored into each gel density of the target proteins to standardize expression of the target proteins.

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