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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.

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.

Table 1
Triplicate C_T values and their means for target and reference genes.

	GAPDH		$GABA_A$ α1		$GABA_A$ α3		$GABA_A$ α3	
	C_T values	C_T mean	C_T values	C_T mean	C_T values	C_T mean	C_T values	C_T mean
Control male	13.31	13.11	16.87	16.53	21.76	21.52	16.19	16.14
	13.10		16.54		21.52		16.10	
	12.91		16.19		21.27		16.14	
Control female	13.13	12.95	15.68	15.61	21.19	21.12	15.25	15.12
	12.78		15.62		21.12		14.98	
	12.96		15.54		21.05		15.11	
d 1 male	13.04	13.01	15.72	15.58	21.58	21.6	15.32	15.28
	13.02		15.44		21.62		15.23	
	12.99		15.59		21.60		15.28	
d 1 female	13.01	12.95	15.73	15.64	21.26	21.17	15.32	15.22
	12.95		15.55		21.07		15.13	
	12.90		15.65		21.17		15.23	
wk 1 male	13.06	13.04	15.78	15.70	21.57	21.5	15.08	15.02
	13.04		15.61		21.43		14.96	
	13.02		15.70		21.50		15.02	
wk 1 female	13.82	13.92	16.05	16.08	21.90	21.91	15.95	16.04
	14.02		16.11		21.92		16.04	
	13.91		16.07		21.91		16.13	
wk 3 male	13.01	12.88	15.34	15.43	21.36	21.22	15.53	15.45
	12.75		15.43		21.07		15.45	
	12.89		15.53		21.22		15.38	
wk 3 female	12.92	12.79	15.43	15.28	21.02	20.95	14.96	14.99
	12.79		15.12		20.89		15.03	
	12.66		15.28		20.95		15.00	

2.4. Assessing DNA fragmentation

Frozen cerebella were sagittally cryosectioned and mounted on slides. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed on cryosectioned-slides at 30 μm thickness using the detection kit, FragEL® (Calbiochem; Cat #QIA38) to determine fragmented DNA in accordance with the manufacturer's guidelines. Cells with damaged DNA appear bright green by fluorescein, and were counted at a magnification of ×100 under a light microscope (Nikon, Eclipse, TE 2000-U) on at least three different slides from two different brains per group.

2.5. Data manipulation and statistics

The statistical analyses were designed to evaluate the effects of genes, times (control, d 1, wk 1, wk 3) and sex (male or female) on mean logarithmic mRNA values represented by mean fold-changes in gene expression for $GABA_A$ Receptor α1, α3, and α6 measured from 24 rat cerebella. Triplicate measures were made on each brain, for a total of 72 observations on each combination. First, mean folds for each gene of interest with respect to the reference gene, GAPDH were calculated, and then standardized to the reference sample (control male). These values were expressed in mean fold changes of standardized mean C_T (threshold cycle) values.

Seventy-two standardized mean fold changes were obtained (Table 2), and the statistical significance of group differences between multiple groups was assessed using an analysis of variance (ANOVA) procedure with *ad hoc* multiple comparisons using Fisher's $LSD_{0.05}$ (least significant difference test calculated at the significance level of 5%) (Armitage and Berry, 1987). The set of TUNEL data was estimated by testing the slope of a linear regression equation ($y = \alpha + \beta x$, where β = slope) on the number of cells with DNA fragmentation. No tendency in apoptotic changes along the time-line would be indicated if $\beta = 0$. No statistical analysis on the results from Western blot assays was attempted, because approximately five brains ($n = 3$–8) per group were pooled; however, the assay was repeated three times, and the most competent results were used for semi-quantification with respect to a reference protein. Data were further normalized to the control male group for visualization of differences among the subgroups.

3. Results

3.1. Real-time RT-PCR

Sequenced PCR products for the genes of interest were precisely superimposed with the published sequences of each. Obtained C_T values and means per group are shown in Table 1. To calculate changes in gene expression, mean folds of gene expression with respect to GAPDH in comparison to male controls were calculated. Mean fold-changes of the genes of interest, $GABA_A$ receptors, were expressed with respect to the corresponding endogenous GAPDH gene, then standardized in percentage to the changes in control males in order to optimize gene expression changes each running. Values in Table 2 are a mean of three fold-changes and a standard error. Fig. 1 that is based on Table 2, demonstrates mean changes of the fold-change values.

The pooled standard error of means (SEM, root mean squared error) was 4.99%. The standard error of mean difference (SE_d) of any comparable pair was $(\sqrt{2}) \times 4.99\% = 7.06\%$. Two means with a difference of more than 14.11% (yielded from $2 \times 7.06\%$, which is the critical mean difference, also called the LSD), are statistically significant at $p < 0.05$. The differences between any two means of the set of data were deemed statistically significant only if the difference was larger than the LSD.

$GABA_A$ α6 expression changes, relative to GAPDH, were significantly affected, particularly in males (Fig. 1c). In males, the amount of fold changes for both α1 and α6 genes significantly increased following IH exposure at d 1 and wk 1 (see Fig. 1a and c). In females, the level of fold changes relative to GAPDH for α1 and α3 was significantly increased after wk 1 exposure, which indicates more transcriptional activity in group wk 1. When differences in gender were considered, fold changes of $GABA_A$ α3 gene in females (fluctuating approximately between 1.136 and 1.321) were significantly elevated compared to those of males (see Fig. 1b). Intermittent hypoxia affected $GABA_A$ α1 gene expression during all stages of development in males compared to controls, while females were most affected by IH only at wk 1. $GABA_A$ α1 gene expressions at baseline and wk 1 were significant higher in females than those of males.

3.2. Western blot of $GABA_A$ receptor subtypes

No noted differences emerged between control and experimental groups in either male or females in $GABA_A$ α1 and $GABA_A$ α3 protein expression (Fig. 2). Expression of $GABA_A$ α6 protein

Table 2
Changes in mRNA relative expression (mean folds) of $GABA_A$ receptors with respect to endogenous GAPDH expression. Mean ± SE which indicates standard error obtained from triplicate measurements.

	Control	d 1	wk 1	wk 3
$GABA_A$ α1				
Male	1 ± 0.138	1.787 ± 0.102	1.675 ± 0.058	1.804 ± 0.069
Female	1.675 ± 0.048	1.636 ± 0.060	2.361 ± 0.029	1.890 ± 0.119
$GABA_A$ α3				
Male	1 ± 0.098	0.878 ± 0.007	0.957 ± 0.027	1.047 ± 0.061
Female	1.175 ± 0.033	1.136 ± 0.043	1.321 ± 0.005	1.175 ± 0.030
$GABA_A$ α6				
Male	1 ± 0.018	1.714 ± 0.030	2.081 ± 0.049	1.383 ± 0.041
Female	1.846 ± 0.099	1.700 ± 0.064	1.886 ± 0.067	1.778 ± 0.025

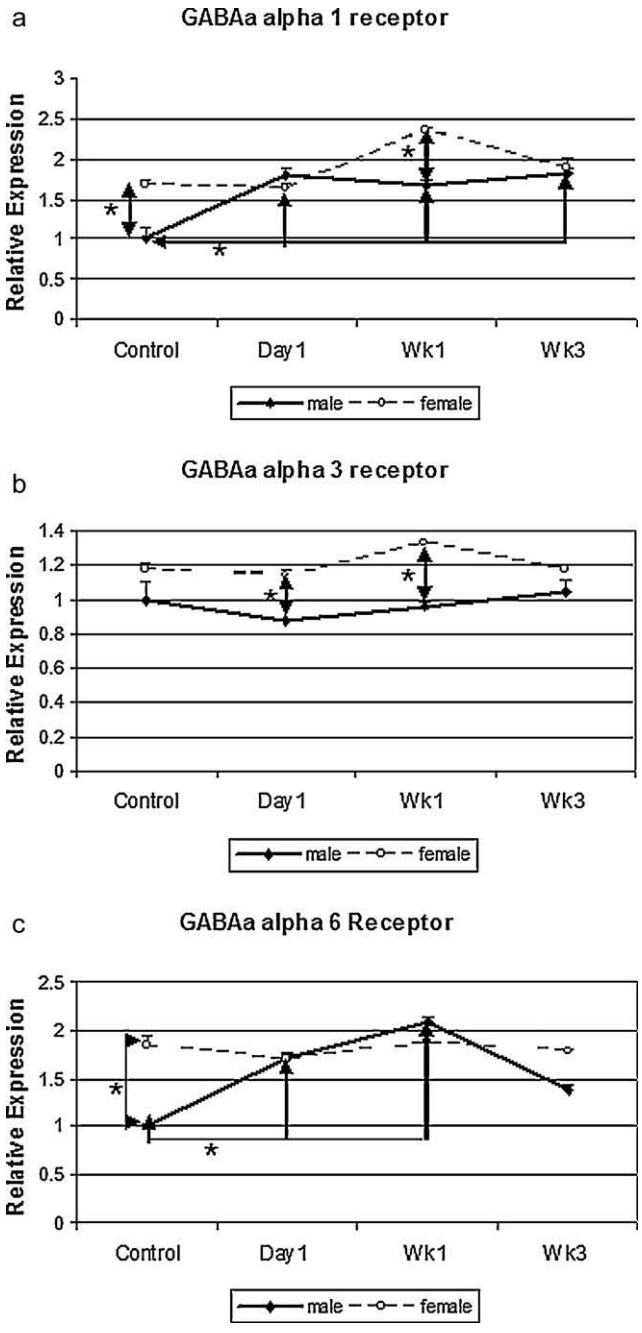

Fig. 1. Comparison of mean fold changes of C_T values for $GABA_A$ α-1,3,6 mRNAs with respect to corresponding GAPDH C_T values. The data are expressed as relative expressions to the endogenous GAPDH gene, and are compared between groups. Asterisks indicate a statistically significant difference at $p < 0.05$.

in female control rats showed slightly lower levels (0.88 of male values). When exposed to 5 h IH, male rats showed consistently decreased $GABA_A$ receptor α6 protein expression levels at all stages of development, compared to controls (d 1, 0.63 of control; wk 1,

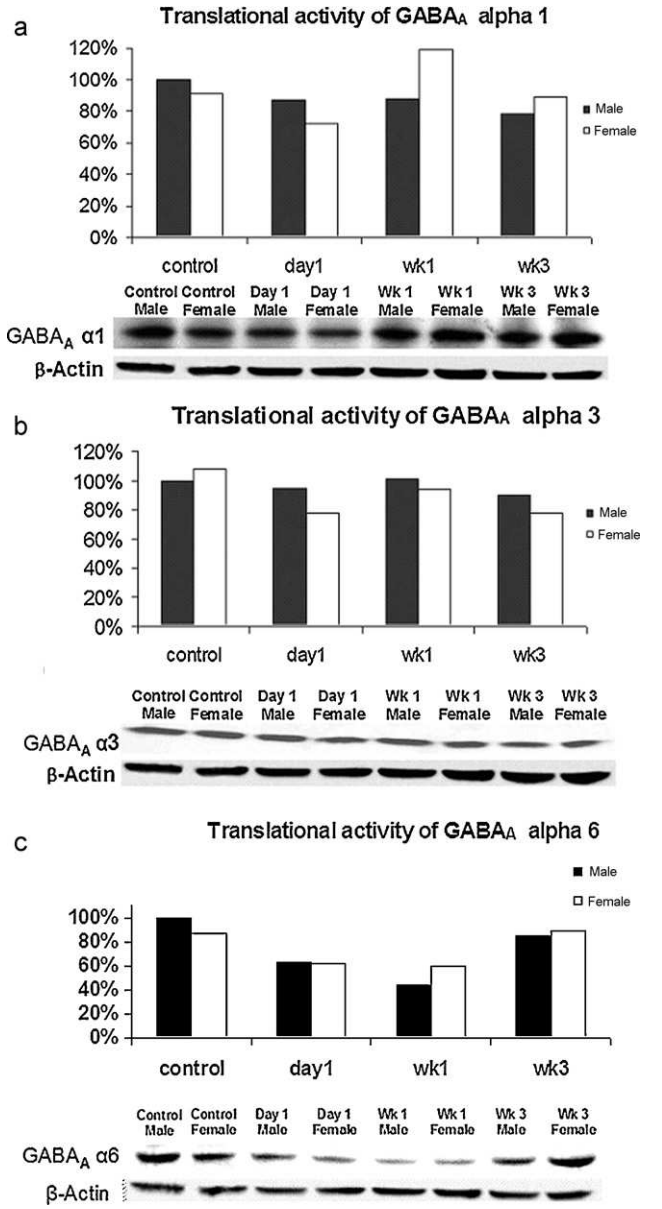

Fig. 2. Western blot assays demonstrate that $GABA_A$ α6 protein levels were markedly reduced after IH insults at wk 1 in both genders. *Y*-Axis denotes that the percentage difference in gel expression of each time point with respect to control male. Note that no statistical analysis on the results was performed.

0.44 of control; wk 3, 0.85 of control. In female rats exposed to IH, a significant decline occurred after d 1 (0.7 of control) and wk 1 (0.68 of control) IH challenge, but no significant differences appeared after wk 3 (1.02 of control).

3.3. DNA fragmentation

Slides stained with FragEL® demonstrated more damage in cells in the group of animals exposed to IH at wk 3, followed by wk 1, d 1 and control groups in both genders. A fluorescein filter allowed cells with DNA fragmentation to appear as light green, indicating positive staining (Fig. 3). Apoptotic cells appeared largely in wk 3 cerebella, followed by wk 1, d 1 and control. This tendency was statistically significant at the level of $p < 0.05$ ($y = 0.211 + 0.975x$) for males and females when evaluating the slope using regression analysis. However, no significant differences were noted between males and females.

4. Discussion

4.1. Postnatal d 7 rat model

Most neonatal hypoxia rat models have used exposure at postnatal d 7, *e.g.*, Holmes et al. (2002), because postnatal d 7 rat model is considered comparable to the neuronal developmental stage of a newborn human infant (McDonald and Johnston, 1990; Rice et al., 1981). In this study, we examined effects of intermittent hypoxic exposure at different ages; postnatal d 1, wk 1 and wk 3, which corresponds to late gestational, newborn and adolescent developmental stages of humans as described by Romijna et al. (1991). These exposure times were selected because potential for hypoxic exposure in infants is maximal in premature and neonatal periods; thus, these times represent conditions more applicable to the human condition.

The results suggest that intermittent hypoxic exposure affects protein concentration of $GABA_A$ receptor α6 in male rats. A significant decrease of $GABA_A$ translational capacity of the α6 subtype in the cerebellum in both genders may result in the affected cerebellum being unable to dampen or coordinate incoming excitatory signals with outgoing signals, an outcome that may contribute to impaired motor coordination, and to specific timing issues, such as aberrant timing of upper airway and diaphragm action as manifested in obstructive sleep apnea. These findings further suggest that perinatal hypoxic insults around birth in the human (equivalent to postnatal wk 1 in rats) could significantly decrease $GABA_A$ α6 receptor expression in the cerebellum, which has the potential to result in life-long alterations in motor, respiratory and sympathetic control, since the cerebellum plays essential roles in such control (Hsieh et al., 2008). Mechanisms underlying the loss of coordination will require further investigation.

Perinatal hypoxia can elicit various short-term, long-term, or life-spanning sequelae (Peyronnet et al., 2000). Early postnatal hypoxic exposure, within the first days of life, induces adverse and long-term effects on the sympathoadrenal system (Soulier et al., 1997), neurobehavioral development (Nyakas et al., 1996), chemoreceptor responses to hypoxia (Hertzberg et al., 1992; Okubo and Mortola, 1988) and development of central catecholaminergic areas involved in respiratory control (Seidler and Slotkin, 1990; Soulier et al., 1997). Prenatal hypoxia also elicits many disturbances which are manifested at, or after birth. Rats born after hypoxic gestation already present at postnatal d 1 with respiratory and metabolic disturbances characteristic of hypoxemia of the newborn (Gleed and Mortola, 1991).

Hypoxia-inducible factor (Hif)-1α may be responsible for these changes. A ubiquitous oxygen monitoring molecule, Hif-1α expression in a cell, increases as the level of oxygen decreases (Semenza, 2011). Increasing Hif-1α induces vascular endothelial growth factor or VEGF expression; thus, VEGF expression level depends on the level of hypoxia. Neural responses of the cerebellum to our IH protocol would differ substantially from those to sustained hypoxia. Response of neuronal cells, such as Purkinje cells of the cerebellum, to sustained hypoxia was adaptive, while degenerative processes appeared following the IH protocol under which the amount of reactive oxygen species (ROS) production increases due to re-oxygenation. The nature of these disturbances appears to depend on the mode, duration, severity of hypoxia, as well as the gestational age of the fetus at the time of insult.

The concept of a transitory, excitatory role of $GABA_A$ receptors in immature neurons, *i.e.*, excitatory rather than inhibitory (Ben-Ari, 2002), would support earlier findings indicating that apoptotic damage, found through TUNEL staining, was most prominent in tissue after IH challenges at 3 wk, while mRNA concentration of $GABA_A$ α1/3 receptors subtypes was highest in wk 1-treated female animals (see Fig. 1). We estimated mean fold changes of the genes of interest with respect to control males results exhibited the same trend as those using each GAPDH C_T value for internal reference. Collectively, the mRNA expression of $GABA_A$ receptors increased (or C_T changes were reduced) after apoptotic damage from IH insults, particularly at wk 1.

4.2. $GABA_A$ receptors and subtypes

Most inhibitory functions of GABA are mediated by $GABA_A$ receptors. In the rat cerebellum, 13 different $GABA_A$ receptor subunits are expressed (Pöltl et al., 2003). The indigenous $GABA_A$ receptor is a heteropentameric chloride channel, comprised of two α (α 1–6), two β (β 1–3), and either a third β, γ (γ 1–3), δ, ρ (ρ 1–2), or an ε subunit (Baumann et al., 2001; Farrar et al., 1999; Klausberger et al., 2001). Depending on their subunit composition, $GABA_A$ receptors show different pharmacological and electrophysiological properties (Sieghart, 2006). The α1 and α6 subtypes are most frequently expressed among the α subunit family, and provide a diverse responsiveness of $GABA_A$ receptors to GABA (Fisher et al., 1997). The α1 subunit is the most abundant α subunit variant in the brain, and is highly expressed throughout most brain regions. In the cerebellum, 75% of all $GABA_A$ receptors contain α1 subunits, and α1 knockout mice show a significant reduction in the total number of $GABA_A$ receptors. Yet, the number of α6-containing $GABA_A$ receptors was unchanged, despite the total number of elevated α6 subunits (Ogris et al., 2006). Likewise, α6 knockout mice showed a strong reduction in the total number of receptors without compensatory up-regulation of other $GABA_A$ receptor subunits, except for a concurrent loss of δ subunits in the α6 knockout cerebellum, suggesting a subunit partnership between α6 and δ subunits (Jones et al., 1997; Nusser et al., 1999). Surprisingly, however, no major phenotypic abnormalities, other than impaired minor motor functions and behavioral alterations have been reported in these knockout animals (Fritschy and Panzanelli, 2006; Jones et al., 1997).

The α6 subunit has a more restricted expression, found only in cerebellar granular cells (Jechlinger et al., 1998) and in the embryologically related granule cells of the cochlear nucleus (Jones et al., 1997; Laurie et al., 1992; Varecka et al., 1994). Several studies investigated the subunit composition of $GABA_A$ receptors containing α6 subunits; however, knowledge is still limited. Approximately 45% of $GABA_A$ receptors extracted from cerebellar extracts of adult rats contain α6 subunits coupled with other subunits, including α1, β1, β2, β3, γ2, and δ as such α6βxγ2, α1α6βxγ2, α6βxδ, or α1α6βxδ (Jechlinger et al., 1998). Although α6 subunit knockout mice reveal no clear phenotypic abnormalities, stargazer mice that lack α6 subunits in the cerebellum show ataxia (Payne et al., 2007). In stargazer

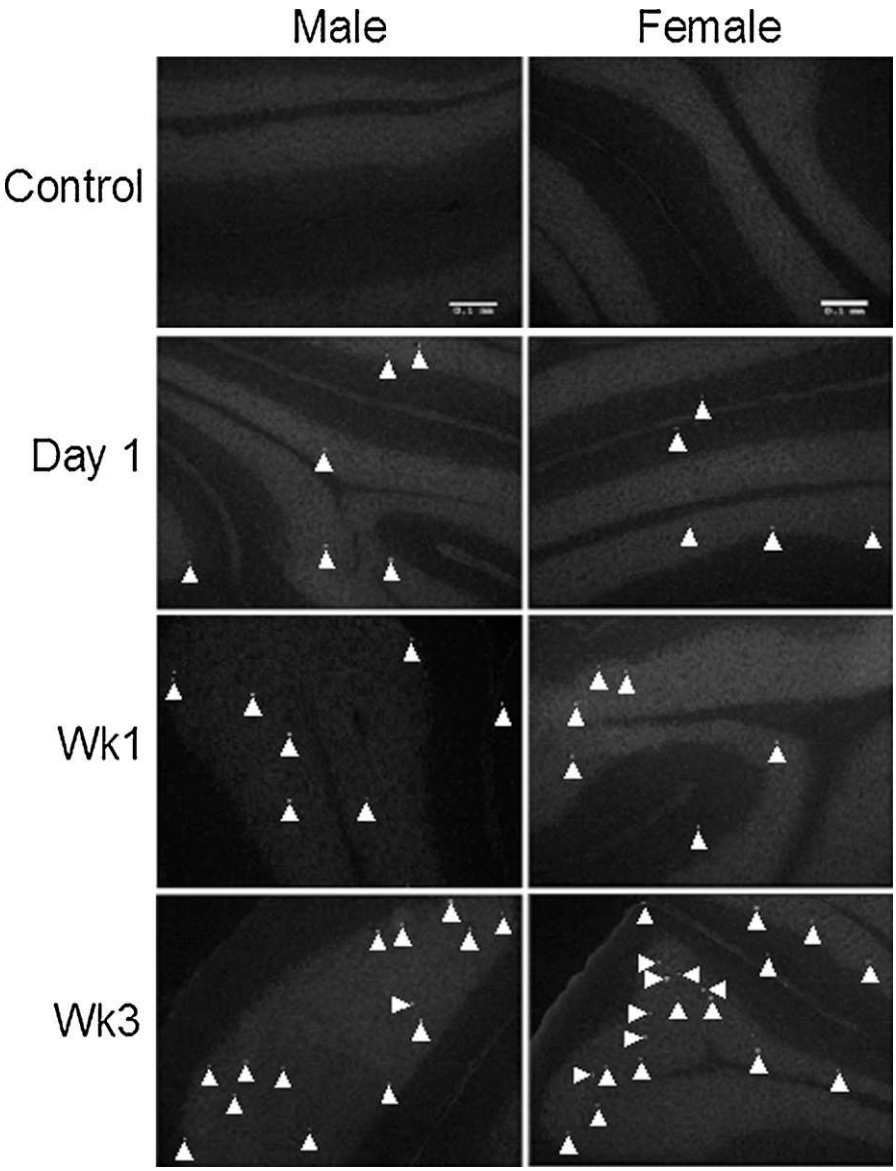

Fig. 3. Neuronal cells with damaged DNA in the cerebellum after 5 h IH insult at d 1, wk 1, and wk 3. Both male and female wk 3 groups showed the greatest numbers of fluorescein-brightened apoptotic cells indicated by arrowheads. Scale bars denote 100 μm.

mice, α6 and δ subunit expressions are reduced by approximately 50% or more of controls. Our results on α6 subunits showed ~50% reduction of protein expression, on average, as well, which may underlie the markedly more-sluggish behaviors from their control counterparts (observations only).

4.3. $GABA_A$ receptors; association with breathing

Previous studies on rats found that the pre-Bötzinger complex, a postulated area of respiratory rhythmogenesis in the medulla (Smith et al., 1991) contains a relatively high level of $GABA_A$ α3 subunits at postnatal d 1, which decline with age, whereas $GABA_A$ α1 subunits were relatively low at postnatal d 1, but increased with age (Liu and Wong-Riley, 2004). The developmental trends of α1 and α3 intersected close to postnatal d 12, a presumed peak critical period of development for brainstem respiratory nuclei (Liu and Wong-Riley, 2004, 2005).

The increase of $GABA_A$ α1 levels of mRNA in female IH-insulted animals at wk 1 was responsible for the increase of corresponding protein. However, the increase of $GABA_A$ α3 levels of mRNA production in female IH-insulted animals at wk 1 will require further investigation, because the protein levels for corresponding mRNA did not significantly change (see Figs. 1 and 2). The protein level may not have been affected because there was inadequate copy of mRNA present to support the protein expression, which may indicate the mRNA level was marginal. Note that the mean C_T level was maintained higher (approximately at 20–21) in $GABA_A$ α3 than those of other subtypes indicating less copy numbers of genes (Table 1). To confirm this speculation, more-substantial insults may be necessary.

Previous studies conversely showed up-regulation in mRNA gene expression, but decreased protein levels of $GABA_A$ receptors after ischemic insults to the rat brain (Neumann-Haefelin et al., 1999), which inferred involvement of a downstream partial translation block in the presence of preserved transcription of the GABA receptor mRNAs for compensation. We also observed a potential partial translation block phenomenon in $GABA_A$ α6 protein levels, despite the significant increase of mRNA level particularly in wk 1 groups, where protein level declines for $GABA_A$ α6 in both genders were vastly greater than mRNA changes. This divergence that

was more clearly shown in male cerebellum samples could result from dysfunction of endoplasmic reticulum, an organelle assembling $GABA_A$ receptors at synaptic sites (Kittler and Moss, 2003; Kneussel, 2002), since the organelle tends to be readily distressed by intermittent hypoxic challenge (Kim et al., 2008b). Zhu et al. (2008) used male mice in a similar setting to demonstrate ER stress burden. They showed persistent up-regulation of markers for ER stress such as CCATT/enhance-binding protein-homologous protein (CHOP) and growth arrest and DNA damage-inducible protein (GADD153).

It is the case that sexual dichotomy in the incidence and expression of some diseases is common, and is found in such diverse conditions as increased male infant risk for breathing control issues related to stress (Genest et al., 2007; Carroll, 2003). The current results show a significant sex-specific difference in the expression pattern change of $GABA_A$ α6 mRNA. As a result, males appear to experience a significantly severe translation block in producing $GABA_A$ α6 protein or ER associated degradation of the protein (Naidoo, 2009). Since we used tissue from the entire cerebellum, it remains uncertain whether disturbances of protein synthesis relative to the level of mRNA in female animals (approximately 20% higher α6 protein than males to wk 1 insult) would indicate a gender disparity. If so, the female cerebellum appears to benefit by some process, which results in a higher expression of $GABA_A$ receptor α6 subunits. These processes appear to maintain homeostasis in neural respiratory motor control better than the male. This speculation remains to be investigated.

In summary, intermittent hypoxia exposure injures the cerebellum in rat pups; the level of apoptosis was most marked immediately after hypoxic exposure, but a reduction in $GABA_A$ α6 receptors appeared most distinctively at 1 wk. Intermittent hypoxia affected protein expression of $GABA_A$ receptor subtype α6 when the rat was exposed one to seven days after birth, but not 3 wk after birth. This clearly indicates that these effects only occur during early development. Therefore, the first 7 postnatal days can be considered as a 'critical window' for neural adaptation in gene regulation process in response to subnormal environment. The alterations resulting from intermittent hypoxia exposure have the potential to modify cerebellar function in later life, thus affecting a large number of motoric, autonomic, and respiratory control activities regulated by this structure. However, the influence of intermittent hypoxia in the perinatal period of females was less than that for males.

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