Respiratory Physiology & Neurobiology 185 (2013) 533–542

Contents lists available at SciVerse ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Chronic, but not acute, ethanol exposure impairs central hypercapnic ventilatory drive in bullfrog tadpoles

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ARTICLE INFO

Article history:
Accepted 12 November 2012

Keywords:
Control of breathing
Alcohol
CO_2
Tadpoles

ABSTRACT

Chronic ethanol exposure early in development is deleterious to neural development and may impair responses to ventilatory stimuli (ventilatory drive) that maintain homeostasis. Central hypercapnic ventilatory drive (CHVD) increases ventilation to ensure pH homeostasis and accommodate the metabolic production of CO_2. We tested the hypothesis that chronic ethanol exposure impairs CHVD in bullfrog tadpoles. Early and late metamorphic tadpoles were exposed in vivo to 0.12–0.06 g/dL ethanol for either 3- or 10-wk durations. Brainstems from these animals were isolated and the neural correlates of ventilation were recorded in vitro during superfusion with normocapnic (1.5% CO_2:98.5% O_2) and hypercapnic (5.0% CO_2:95.0% O_2) artificial cerebral spinal fluid. Normocapnic neuroventilation was unaffected by chronic ethanol exposure. The typical response to hypercapnia, an increase in lung burst frequency, was lost following 10 but not 3 wk of ethanol exposure in both early and late metamorphic tadpoles. The neuroventilatory effects of chronic ethanol exposure were distinguishable from those of acute central ethanol (0.08 g/dL) exposure, which attenuated early metamorphic tadpole normocapnic neuroventilation, but had no effect on tadpole CHVD. Thus, 10 wk of ethanol exposure both early and late in metamorphosis impairs CHVD in bullfrog tadpoles.

1. Introduction

Breathing is a vital homeostatic process modulated primarily by the need to neutralize the acidosis created by CO_2 produced by the catabolism of metabolic fuels (Nattie, 1999; McCrimmon et al., 2000; Feldman et al., 2003). Central hypercapnic acidosis elicits an increase in the rate of ventilation to expedite exhalation of CO_2 (O'Regan and Majcherczyk, 1982; Milsom, 1995; Nattie, 1999; Putnam et al., 2004, 2005). Responsiveness to another ventilatory stressor, hypoxia, is decreased following chronic developmental ethanol exposure in rats (Dubois et al., 2008; Kervern et al., 2008). Fewer studies have looked at the effect of ethanol on hypercapnic responses and CHVD, despite evidence that ethanol's action on GABA neurotransmission may alter GABA's alleged role in disinhibiting responses to hypercapnia (Peano et al., 1992; Horn and Waldrop, 1994; Gourine and Spyer, 2001; Liu et al., 2003; Kuribayashi et al., 2008). Acute ethanol lowers the response threshold of CHVD in adult humans but acute, late-gestational exposure has no impact on fetal hypercapnic drive (Sahn et al., 1975; Michiels et al., 1983; Marchal and Droulle, 1988). Investigating the effect of chronic ethanol exposure on responsiveness of the respiratory neural circuit to the stressor, hypercapnia, could provide important background for investigations of the mechanisms that underlie central CO_2 sensitivity.

The ventilatory CO_2 response is a homeostatic control mechanism to maintain optimal physiological pH. CO_2 is a by-product of cellular fuel catabolism and, if allowed to accumulate, would cause tissue acidosis. In some species respiratory CO_2 sensitivity changes with development but it has not been investigated across development in a great many species. CO_2 sensitivity is low in newborn mice and dogs, then increases to adult levels in the post-natal period; it is high in newborn rats and pigs, drops over the first 1–2 wk of development, and then increases to adult levels in the post-natal period (for review see Putnam et al., 2005). In humans, however, the respiratory CO_2 response is consistent from full-term birth to adulthood (Sovik and Lossius, 2004). We identified consistent central chemosensitive sites and CO_2 sensitivity in tadpoles and frogs (Taylor et al., 2003a,b). Developmental consistency in respiratory CO_2 sensitivity is a significant parallel between frogs and humans, one that does not exist between humans and traditional mammalian research animals.

Ethanol is a neuroteratogen that causes a wide range of adverse effects on development including central nervous system dysfunctions comprising cognitive impairments, behavioral disturbances, and neurological damage (Clarren et al., 1978). Prenatal ethanol

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http://dx.doi.org/10.1016/j.resp.2012.11.006

exposure is associated with alterations in autonomic control that occur during sleep soon after birth (Fifer et al., 2009). The neuroventilatory consequences of developmental ethanol exposure depend on the timing of exposure relative to development. Acute ethanol exposure nearly abolishes fetal breathing movements in late but not early gestation (Goodlett and Eilers, 1997). Chronic late-gestational ethanol exposure results in numerous neuronal deficits (Marchal and Droulle, 1988; Smith et al., 1990; Brien and Smith, 1991; Hamre and West, 1993; Marcussen et al., 1994; Watson et al., 1996; Goodlett and Eilers, 1997; Goodlett et al., 1998), and early gestational ethanol exposure as a result of maternal binge drinking confers the greatest risk of neonatal breathing pathologies (Iyasu et al., 2002). Ethanol exposure during different periods of brain development results in regional differences in cell loss (West et al., 1990; Maier et al., 1999), and this could account for disparate ethanol vulnerability, based on developmental timing and duration of exposure, of the neural network controlling breathing.

To our knowledge the only evaluation of the consequences of chronic developmental ethanol exposure (i.e., exposure to a constant moderate dose of ethanol for a period of several weeks) on CHVD was done in early metamorphic bullfrog tadpoles (Taylor et al., 2008). Developing bullfrog tadpoles frequently have been used as a model to evaluate the consequences of teratogen exposures (Beatty et al., 1976; Neal et al., 1979; Schuytema et al., 1991; Brundage and Taylor, 2009; Brundage et al., 2010). Bullfrog tadpoles were used in this research to capitalize on the advantages of the frog isolated brainstem preparation for use in developmental neurophysiology. The isolated frog brainstem provides, at any stage of development, the intact and functional central circuit that controls breathing, and it allows long-term investigation of central respiratory CO_2 responses under physiologically relevant conditions. Questions have been raised as to the relevance of CO_2 as a respiratory signal in fully aquatic tadpole stages; however, we have shown (Taylor et al., 2008) that such tadpoles respond to hypercapnic exposure with a significant increase in their surfacing, presumably air-breathing, events. Here we investigate the effect of chronic ethanol exposure on a respiratory neural circuit that responds to CO_2, with the expectation that this will provide insight into the character of CO_2 sensitivity in a respiratory neural circuit. In both in vivo and in vitro experiments, tadpoles continuously exposed to 0.1% ethanol (v/v) for 8–12 wk throughout development failed to respond normally to hypercapnic challenges (Taylor et al., 2008). It was unclear, however, whether this response was based on the developmental stage of the tadpoles, the duration of ethanol exposure, or an acute reaction to ethanol. The present study was designed to clarify these issues. Tadpoles from both early and late periods of metamorphosis (i.e., before and after, respectively, the lungs become the primary site of respiratory gas exchange) were exposed to ethanol for either 3- or 10-wk durations prior to assessing CHVD in their isolated brainstems. Responses were compared with control tadpoles and those of brainstems acutely exposed to ethanol via bath application. We predicted that early metamorphic tadpoles would demonstrate an increased susceptibility to chronic ethanol exposure, and that ethanol effects on CHVD in early metamorphic tadpoles would be distinct from those in late metamorphic tadpoles and brainstems acutely exposed to ethanol.

2. Methods

2.1. Animals

Studies were performed on bullfrog tadpoles (*Lithobates catesbeianus*; $n = 108$) purchased from a commercial supplier (Sullivan Co. Inc., www.researchamphibians.com). Tadpoles were maintained at room temperature and were fed goldfish food daily. Tadpoles were housed for 10 wk in aquaria with either dechlorinated water only or dechlorinated water containing ethanol (0.12–0.06 g/dL). The ethanol concentration varied due to the volatilization of ethanol from the tank. This range encompasses the concentration of a previous study using bullfrogs (Taylor et al., 2008) and the 0.08 g/dL blood alcohol content that is the legal definition of alcohol intoxication in many western countries (Caldeira et al., 2004). 3-wk ethanol exposure was achieved by maintaining tadpoles in dechlorinated water for 7 wk before transferring them to ethanol-containing water for 3 wk. Consequently all animals were maintained in the laboratory for 10 wk regardless of the duration of ethanol exposure. Taylor et al. (2008) reported impairments of hypercapnic response after 8–12 wk of ethanol exposure. We wanted to reproduce those findings and determine if CHVD impairment is stage- or duration-dependent; thus, the mid-range 10-wk exposure was selected. 3-wk ethanol exposure was chosen to determine whether a significantly shorter exposure could lead to a similar impairment.

The developmental stage of each tadpole was determined twice, once at the start of treatment and again at the time of dissection to ensure developmental homogeneity of the treatment groups. At the time of dissection each tadpole was either early metamorphic (forelimbs absent, hind limbs paddle-like without joints or separated toes) or late metamorphic (forelimbs and hind limbs present, tail being resorbed), which corresponded to developmental stages 7–12 or 20–25, respectively, in the classification scheme of Taylor and Kollros (1946). Tadpoles included in early metamorphic groups were stages 7–9 at the start of their 10-wk laboratory maintenance, while those included in late metamorphic groups were stages 18–20. This was true for animals that received either 3 or 10 wk of chronic ethanol exposure, as they were all maintained for 10 wk. Four animals that did not remain within these stage ranges were excluded from the datasets. Since this is a small proportion of the study population that was also distributed across multiple treatment groups, we have no evidence to suggest a confounding influence of ethanol treatments on developmental progression. The ethanol-exposed animals appeared to develop and grow similarly and at a similar pace compared with unexposed animals, although development and growth were not specifically quantified for individual animals, as the effect of ethanol on whole-animal development was not our focus. All care and experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Alaska Fairbanks and complied with all state and federal ethical guidelines.

2.2. Surgical preparation

Each tadpole was anesthetized by immersion for 1–2 min in cold (4 °C) 0.2 mM tricaine methanesulfate (MS222; Sigma, www.sigmaaldrich.com) in dechlorinated water buffered to pH 7.8 with $NaHCO_3$. The front of the head rostral to the nares and the back of the body (hind limbs and tail, if present) were removed. The dorsal cranium and forebrain rostral to the diencephalon were resected and the fourth ventricle opened by removing the choroid plexus. The remaining brainstem and spinal cord were removed *en bloc* and further trimmed rostrally to the optic tectum and caudally to the brachial nerve. During dissection, exposed tissues were superfused with cold artificial cerebral spinal fluid (aCSF) composed of (in mM) 104 NaCl, 4 KCl, 1.4 $MgCl_2$, 10 D-glucose, 25 $NaHCO_3$ and 2.4 $CaCl_2$ equilibrated with 100% O_2. The aCSF HCO_3^- concentration is similar to that of plasma from late metamorphic tadpoles and frogs but higher than that in plasma from early metamorphic tadpoles (Just et al., 1973). This HCO_3^- concentration has been used in previous tadpole studies (Taylor et al., 2003a,b, 2008) and was selected here to ensure comparability with previous