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Developmental alcohol exposure disrupts circadian regulation of BDNF in the rat suprachiasmatic nucleus

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Abstract

In rats, damage to neuronal populations in some brain regions occurs in response to neonatal alcohol exposure coinciding with the period of rapid brain growth. These alcohol-induced defects in brain development may persist into adulthood and thus have long-term implications for the functional characteristics of damaged neuronal populations. The present study examined the effects of neonatal alcohol exposure on endogenous rhythmicity of the circadian clock located in the rat suprachiasmatic nucleus (SCN). Specifically, experiments were conducted to determine whether neonatal alcohol exposure alters the circadian rhythm of brain-derived neurotrophic factor (BDNF) content in the rat SCN because this neurotrophin is an important rhythmic output from the SCN clock. Male rat pups were exposed to alcohol (4.5 g/kg/day) or isocaloric milk formula on postnatal days 4-9 using artificial rearing methods. At 5-6 months of age, SCN and hippocampal tissue was harvested and subsequently examined for content of BDNF protein. Time-dependent fluctuations in BDNF protein levels were assessed by enzyme-linked immunosorbent assay (ELISA). In alcohol-treated rats, SCN levels of BDNF were significantly decreased and were characterized by a loss of circadian rhythmicity relative to those observed in control animals. In comparison, hippocampal levels of BDNF displayed no evidence of circadian regulation in all three treatment groups, but were slightly lower in alcohol-treated animals than in control groups. Importantly, these observations suggest that alcohol exposure during the period of rapid brain development may cause permanent changes in the SCN circadian clock.

Keywords

Circadian rhythms; Clock; Ethanol; Neurotrophins; Brain-derived neurotrophic factor; Hypothalamus; Hippocampus

1. Introduction

During critical stages of brain development, neuronal populations in different regions are vulnerable to profound damage by alcohol exposure. With regard to brain development, alcohol exposure during the brain growth spurt (early postnatal period), which corresponds to the third trimester of gestation in humans, disrupts normal neural development within the hippocampus, cerebellum, and olfactory bulb [2,3,5,6]. Because accelerated synaptogenesis, neuronal proliferation, and myelination occur during this temporal window, regional damage associated with developmental alcohol exposure is often marked by delayed patterns of differentiation, cell loss, altered neuronal circuitry, or decreased expression of neurochemical signals such as

neurotrophins [9,10,23]. These alcohol-induced defects in brain development persist into adulthood and thus have long-term implications for the functional characteristics of damaged neuronal populations. For example, early postnatal alcohol exposure in the rat produces cell loss in discrete areas of the cerebellum and hippocampus that are respectively associated with deficits in motor performance and learning/memory [15,32]. However, information on the full extent of the permanent damage to specific brain regions and associated functional disturbances caused by developmental alcohol exposure are still emerging.

Limited information is available on whether the damaging effects of alcohol exposure during brain development also impinge upon the neural substrate responsible for the circadian regulation of physiological processes and behavior [13]. In mammals, the suprachiasmatic nucleus (SCN) of the anterior hypothalamus functions as an internal biological clock governing the generation and photoentrainment of circadian rhythms in a variety of biological processes [25]. Consistent with SCN pacemaker function in regulating circadian rhythmicity in other cells and tissues, many of its molecular, biochemical, and physiological activities oscillate independent of environmental or external input. Circadian rhythms in gene expression, the levels of various neuropeptides and neurotransmitters, cellular metabolism, and neuronal firing rate are indigenous to the SCN in vivo and persist following isolation of SCN cells in vitro [17]. Some of these endogenous oscillations are thought to provide important output signals that mediate the generation of overt circadian rhythmicity in other brain regions or peripheral organs. Rhythmic output signals from the SCN can also serve to modulate clock input that synchronizes or entrains circadian rhythms. For example, the rhythmic regulation of the neurotrophin, brain-derived neurotrophic factor (BDNF), in the SCN is an important process in the circadian sensitivity of the clock mechanism to photic input [19,20].

Because clinical sleep-wake disturbances have been observed in human neonates, children, and adolescents following prenatal exposure to alcohol [27,30,31], the present study used a rat model to determine whether alcohol exposure during the brain growth spurt also produces long-term disturbances in the circadian timekeeping function of the SCN. Based on evidence indicating that alcohol exposure decreases BDNF expression in other regions of the developing rat brain [8,21,22], experiments specifically examined the effects of neonatal alcohol exposure on the circadian rhythm of BDNF content in the SCN of adult rats.

2. Methods

2.1. Subjects

The subjects were 60 male Sprague-Dawley rat pups derived from 15 time-mated litters. The animals were born and reared in the vivarium at the Texas A&M University System Health Science Center under a standard 12-h light:12-h dark photoperiod (LD 12:12; lights-on at 0600 h). On postnatal day (PD) 1 (date of birth designated as PD 0), the newborns within each litter were culled to 8-10 pups per litter, utilizing cross-fostering procedures as necessary. On PD 4, the rat pups were randomly assigned to one of the two artificial rearing treatment groups receiving either alcohol (EtOH, 4.5 g/kg/day, $n = 20$) or maltose-dextrin (gastrostomy control [GC], 0 g/kg/day, $n = 20$) from PD 4 through 9, and one normal suckle control group (SC, $n = 20$). The SC group was included to control any effect produced by artificial rearing methods. In order to minimize the potential confound of litter effects, no more than two pups from the same litter were assigned to the same treatment condition.

2.2. Artificial rearing procedure and animal housing

The artificial rearing process has been described previously in detail [34]. Briefly, on PD 4, gastrostomy tubes were inserted down the esophagus under Metofane anesthesia and implanted into the stomachs of pups assigned to the artificially reared groups [11,33]. From PDs 4 to 9,

pups were maintained in the artificial rearing apparatus and received their daily nutritional requirements through formula (diet)-filled syringes which were operated by a timer-activated infusion pump (Model# 935, Harvard Apparatus, Holliston, MA). The formula was provided daily in twelve 20-min fractions (i.e., every 2 h). Gastrotomized pups received alcohol treatment or isocaloric maltose-dextrin solution from PDs 4 to 9. Following this treatment regimen, pups were maintained on the artificial rearing apparatus for three additional days with regular diet (maltose-dextrin with no alcohol) to allow alcohol withdrawal in the EtOH group. On PD 12, artificially reared pups were fostered to a lactating dam after their gastrotomy tubes had been cut and sealed. All pups were coded by injecting india ink to their paw for future identification [14]. Animals were weaned on PD 21 and housed two to three per cage. Access to food and water was provided ad libitum for the remainder of the experiment. Daily animal care was performed at random times.

2.3. Drug administration

Beginning around midday of the LD 12:12 photoperiod (1200 h) from PDs 4 and 9, alcohol (10.2%, v/v) was administered to the EtOH group through two of the daily 12 feedings at the dose of 4.5 g/kg/day. This dose of alcohol produces neuronal loss in various developing brain regions [2,3,5,6]. The GC group (0 g/kg/day alcohol) was given the appropriate concentration of isocaloric maltose-dextrin solution in place of alcohol.

2.4. Blood alcohol concentration

Peak blood alcohol concentrations (BACs) for all alcohol-treated pups were measured using a gas chromatograph (Model# 3400, Varian, Palo Alto, CA). Twenty microliter tail blood samples were drawn 90 min after the beginning of the second alcohol feeding on PD 6 (peak BAC) and 30 min prior to the first alcohol feeding on PD 7 (trough BAC) [1,16].

3. SCN and hippocampal tissue collection

At approximately 5 months of age, animals were housed individually in cages equipped with running wheels and maintained on LD 12:12 until their activity rhythms showed stable entrainment ($\approx$ 14-21 days) such that the daily onset of activity occurred shortly after lights-off (at 1800 h). Beginning at the offset of this photoperiod (circadian time [CT] 12), animals were exposed to constant darkness (DD). Sixteen hours later (1000 h or CT 4), animals from the GC, SC, and EtOH groups were sacrificed at 6-h intervals ($n = 4$) for 24 h by decapitation under dim red light (Kodak filter GBX-2). Thus, brain tissue was collected during the subjective day (i.e., when the light phase would have occurred in the previous LD 12:12 photoperiod) at CT 4 and 10, during the subjective night (i.e., coinciding with previous dark phase or the animal's active period) at CT 16 and 22 and again during the next circadian cycle at CT 4. The SCN was immediately dissected as described previously [12,19]. Briefly, the entire brain was removed and sectioned in the coronal plane at the mid-level of the optic chiasm (600-800 μ m). The SCN was obtained by dissecting a small square of tissue in the ventral extent of the brain section adjacent to the third ventricle. Following excision of the SCN, the rest of the brain was blocked in the coronal plane and the hippocampus was excised along its rostral-caudal extent to analyze the effects of postnatal alcohol exposure on BDNF levels in a brain region characterized by nonrhythmic expression [19]. Tissue samples were frozen in liquid nitrogen and stored at -80 °C until subsequent assay for BDNF content.

3.1. BDNF enzyme-linked immunosorbent assay

For analysis of BDNF protein content by enzyme-linked immunosorbent assay (ELISA), SCN and hippocampal tissue from each individual animal was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA). Soluble proteins were recovered from the organic phase by

sequential precipitation [7] and dissolved in 1% SDS. Prior to ELISA analysis, protein content in each sample was determined by the bicinchoninic acid method (Pierce, Rockford, IL).

BDNF content in recovered protein samples was determined using an ELISA sandwich protocol. Ninety six-well microplates (Nunc, Rochester, NY) were coated overnight at 4 °C with 100 µl of rabbit anti-BDNF antibody (Santa Cruz Biotech, Santa Cruz, CA) diluted (1:250) in 0.025 M carbonate-bicarbonate buffer (pH 9.7). With interceding washes (50 mM Tris-buffered saline [TBS], pH 7.4), the plates were subjected to sequential 2-h incubations at room temperature with blocking solution (1% bovine serum albumin in TBS), triplicate aliquots of tissue protein samples or BDNF standards (0.1-100 ng/well), chicken anti-BDNF antibody (1:500; Promega, Madison, WI) and alkaline phosphatase-conjugated goat anti-chicken IgY (1:1000, Promega). Alkaline phosphatase activity was detected using *p*-nitrophenyl phosphate (Pierce) dissolved in 0.1 M bicarbonate buffer (pH 10) as the color substrate. After color development (40-50 min), absorbance at 405 nm was measured using a plate reader (Bio-Tech, Winooski, VT). Using serial dilutions of known amounts of recombinant human BDNF, this color reaction yielded a linear standard curve from 1 to 250 ng/ml. The intra- and interassay coefficients of variation were less than 5% and 10%, respectively. The content of BDNF in tissue lysates was quantified within the linear range of a standard curve and normalized for the soluble protein (Ag) that was assayed in each sample. The rabbit and chicken anti-BDNF antibodies used in this assay show less than 2% cross-reactivity with NGF, NT-3 or NT-4 (at concentrations as high as 10 µg/ml). Specificity of these antibodies for BDNF has also been verified previously by Western blot analysis [18].

3.2. Statistical analyses

BDNF protein levels in the SCN and hippocampus were analyzed using two-way analyses of variance (ANOVAs) with treatment (EtOH vs. GC vs. SC) and time (CT 4, 10, 16, 22, 28) as two independent variables. Fisher's PLSD post hoc analyses were used if significant main effects were obtained. To identify rhythmic variation in BDNF protein levels in the SCN and hippocampus, time-point determinations within a treatment group were evaluated using one-way ANOVA irrespective of the presence or absence of significant interaction between treatment and time factors. Fisher's PLSD post hoc analyses, if required, were applied to determine the loci of significance in these analyses. The α value was set at .05 for all statistical analyses.

4. Results

4.1. Blood alcohol concentration analysis

The administration of alcohol in two consecutive feedings out of the 12 daily feedings resulted in peak BACs on PD 6 ranging from 272.0 to 380.0 mg/dl. The mean peak BAC was 333.47 ± 11.8 mg/dl. These BAC values are similar to those observed in previous studies using the same alcohol dosage [26].

4.2. ELISA: BDNF protein analysis

Developmental alcohol exposure altered both the relative levels and the temporal profile of BDNF content in the SCN (Fig. 1A). Two-way ANOVA analysis conducted on the SCN BDNF data yielded not only the main effects of treatment [$F(2,45) = 98.73$, $P < 0.01$] and time [$F(4,45) = 12.90$, $P < 0.01$], but the interaction between these factors [$F(8,45) = 3.05$, $P < 0.01$]. Post hoc analysis (Fisher's PLSD) performed on the main effect of treatment showed that BDNF levels in the SCN were significantly lower ($P < 0.01$) in the EtOH group compared with those in the GC and SC groups. The significant interaction between treatment and time was examined further using separate one-way ANOVAs. This analysis revealed that SCN content of BDNF in the EtOH group was significantly lower (all at $P < 0.01$) than those in the GC and

SC groups at all time points. To evaluate the temporal patterns of BDNF content in the SCN for evidence of circadian rhythmicity, separate one-way ANOVAs were conducted across all time points for each treatment condition. These analyses indicated that BDNF protein levels in the SCN fluctuated significantly over time in both control groups [GC: $F(4,15) = 8.81$; SC: $F(4,15) = 11.01$, $P < 0.01$], but not in the EtOH group. Within the GC and SC groups, post hoc analysis (Fisher's PLSD) indicated that the mean levels of BDNF protein in the SCN during the subjective night at CT 16 and 22 were significantly greater ($P < 0.01$) than those observed during the subjective day at CT 4 and 10. Similar to the rhythmic pattern observed previously in the rat SCN [19,20], BDNF protein levels in both control groups oscillated such that SCN content was 2-fold higher during the subjective night than during the subjective day. In summary, rats exposed to developmental alcohol were distinguished a marked decrease and loss of rhythmicity in SCN levels of BDNF protein relative those found in control animals.

In contrast to the SCN, hippocampal levels of BDNF showed no evidence of circadian or even regular rhythmic variation within control and alcohol-exposed groups (Fig. 1B). Analysis of hippocampal BDNF data with a two-way ANOVA did not show a main effect of time or an interaction between treatment and time factors. However, the overall analysis resulted in a main effect of treatment [$F(2,45) = 4.63$, $P < 0.05$]. Post hoc analysis (Fisher's PLSD) indicated that hippocampal BDNF levels at all circadian times were significantly lower ($P < 0.05$) in the EtOH group than in the GC group. Although hippocampal BDNF levels in the EtOH group were reduced compared with those in the SC group, the differences did not reach the criterion level of statistical significance ($P = 0.077$).

5. Discussion

The present study demonstrates that early postnatal alcohol exposure disrupts endogenous rhythmic properties of the SCN circadian clock in adult rats. Similar to the circadian pattern observed previously in rats [19], BDNF content within the SCN oscillated rhythmically in GC and SC animals such that relative levels were low during the subjective day (CT 0-12) and high during the subjective night (CT 12-24). In the SCN of alcohol-treated rats, BDNF levels were decreased in comparison with both control groups and distinguished by a loss of circadian rhythmicity. Hippocampal content of BDNF exhibited no sign of circadian rhythmicity in GC, SC, and EtOH animals, although levels of this neurotrophin in alcohol-treated rats were lower than those in both control groups. The observed decreases in BDNF content within the SCN and hippocampus are consistent with previous reports demonstrating that BDNF levels in specific brain regions are reduced following prenatal or early postnatal alcohol exposure [8, 21,22], although these studies examined the acute effects of alcohol on brain development. Consequently, the present observations suggest that alcohol-induced decreases in BDNF levels such as those found in the hippocampus and hypothalamus may be permanent and thus may impact upon the function of these brain regions in adulthood.

The specific effects of reduced BDNF content and rhythmicity on SCN circadian function in alcohol-treated animals are unknown. The rhythmic regulation of BDNF levels in the SCN has been implicated in the mechanism by which the circadian clock synchronizes or entrains to photic signals conveyed by the retinohypothalamic tract (RHT). Similar to its role in modulating synaptic transmission within the visual cortex [4], BDNF is thought to mediate the circadian sensitivity of the SCN to the resetting or phase-shifting effects of light by enhancing synaptic input via the RHT [20]. In the rat SCN, the rhythmic elevation of BDNF-mediated signaling during the subjective night represents a critical process in the light-induced resetting of the circadian clock. When SCN levels of BDNF are endogenously low during subjective day, the clock mechanism is insensitive to the phase-shifting effects of light. A role for BDNF in the photic regulation of the SCN is also supported by anatomical evidence for the close apposition between BDNF-expressing cells in the ventrolateral SCN and TrkB-

immunoreactive fibers emanating from the optic chiasm [18]. In addition to its role in the circadian regulation of SCN sensitivity to light, BDNF may also be involved in modulating local cellular interactions or coupling between autonomous clock cells in the SCN. The possible role of BDNF-mediated signaling in the synchronization of independent clock cells is supported by anatomical observations indicating that many BDNF-expressing cells in the medial SCN are located adjacent to TrkB-immunopositive perikarya [18].

Based on these implications for normal BDNF function in the SCN, the alcohol-induced disruption of the rhythmic regulation of this neurotrophin would be expected to affect the circadian regulation of the clock mechanism by light-dark signals and/or synchronization of autonomous rhythmicity among individual clock cells in the SCN. Specifically, the long-term damping of BDNF rhythmicity induced by early postnatal alcohol exposure may alter the entrainment and phase-shifting responses of circadian behavioral rhythms to light-dark cues or induce perturbations in the normal periodicity of these rhythms. It is noteworthy that BDNF-deficient mutant mice show congruous changes in the circadian regulation of wheel-running behavior. In mice heterozygous for the BDNF null mutation, deficits and damped rhythmicity in SCN levels of this neurotrophin are accompanied by marked decreases in light-induced phase shifts and circadian period of the activity rhythm [20]. In addition, the long-term consequences of alcohol exposure during early brain development on SCN circadian function have been directly observed in a recent study demonstrating that prenatal alcohol treatment alters the light-dark entrainment of the body temperature rhythm in adult rats [28]. Similar to this finding, our preliminary analysis of circadian wheel-running behavior suggests that the patterns of entrainment to light-dark cycles may be unstable or altered in adult rats exposed to alcohol during the early postnatal period [24]. Taken together, these results suggest that alcohol exposure during early brain development has direct and lasting effects upon the circadian clock and its regulation of circadian rhythms.

Although specific mechanisms for alcohol-induced alterations in circadian clock function were not addressed in the present study, the observed disruption of an important circadian output of the SCN such as rhythmic BDNF expression may reflect damage to critical elements of the molecular clockworks. In mammals, the “gears” of central clock mechanism consists of genes whose products oscillate with a circadian periodicity and interact within an interlocked transcriptional/translational feedback loop [29]. Specifically, *Clock*, *Bmal1* (*Mop3*), *Period1* (*Per1*), *Per2*, *Cryptochrome1* (*Cry1*), and *Cry2* are thought to represent core components of this molecular feedback loop because mutation or knockout of these genes in mice alters or abolishes the circadian rhythm of activity. Thus, postnatal alcohol exposure may directly impair the rhythmic regulation of these core clock elements and thereby interfere with the endogenous capacity of the SCN to generate circadian output signals. In this case, alcohol-induced disturbances within the clock mechanism itself should similarly affect other endogenous indices of SCN rhythmicity. Alternatively, alcohol exposure during early brain development may compromise BDNF rhythmicity by affecting key “upstream” elements responsible for the circadian regulation of this SCN output signal. Further analysis will be necessary to elucidate the basic mechanisms for the long-term impact of postnatal alcohol exposure on SCN circadian function. Nevertheless, our results strongly suggest that the alterations in SCN content and rhythmic regulation of BDNF may reflect important developmental and circadian rhythm anomalies associated with early postnatal exposure to alcohol.

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Fig. 1. Temporal pattern of BDNF protein content in the SCN (A) and hippocampus (B) of suckle control (SC, ■), gastrostomy control (GC, ●), and alcohol-treated (EtOH, △) rats sacrificed at 6-h intervals during exposure to constant darkness. Samples were specifically collected during the subjective day at CT 4 and 10 (i.e., 4 and 10 h after when lights-on would have occurred in the previous LD 12:12 photoperiod), during the subjective night at CT 16 and 22, and again during the next circadian cycle at CT 4. Symbols represent the mean ($\pm$ S.E.M.) values for BDNF protein ($n = 4$) normalized for soluble protein content (μ g). Asterisks indicate circadian

times during which SCN levels of BDNF were significantly greater ($P < 0.01$) in comparison to those observed at CT 4 and 10.