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Effects of neonatal alcohol exposure on vasoactive intestinal polypeptide neurons in the rat suprachiasmatic nucleus

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Abstract

Neonatal alcohol exposure produces long-term changes in the suprachiasmatic nucleus (SCN) that are presumably responsible for disturbances in the light–dark regulation of circadian behavior in adult rats, including the pattern of photoentrainment, rate of re-entrainment to shifted light–dark cycles, and phase-shifting responses to light. Because SCN neurons containing vasoactive intestinal polypeptide (VIP) receive direct photic input via the retinohypothalamic tract and thus play an important role in the circadian regulation of the SCN clock mechanism by light, the present study examined the long-term effects of neonatal alcohol exposure on VIP neuronal populations within the SCN of adult rats. Male Sprague-Dawley rat pups were exposed to alcohol (EtOH; 3.0, 4.5, or 6.0 g/kg/day) or isocaloric milk formula (gastrostomy control; GC) on postnatal days 4–9 using artificial-rearing methods. At 2–3 months of age, animals from the suckle control (SC), GC, and EtOH groups were exposed to constant darkness (DD) and SCN tissue was harvested for subsequent analysis of either VIP mRNA expression by quantitative polymerase chain reaction (PCR) and in situ hybridization or of VIP-immunoreactive (ir) neurons using stereological methods. Neonatal alcohol exposure had no impact on VIP mRNA expression but dramatically altered immunostaining of neurons containing this peptide within the SCN of adult rats. The relative abundance of VIP mRNA and anatomical distribution of neurons expressing this transcript were similar among all control- and EtOH-treated groups. However, the total number and density of VIP-ir neurons within the SCN were significantly decreased by about 35% in rats exposed to alcohol at a dose of 6.0 g/kg/day relative to that observed in both control groups. These results demonstrate that VIP neuronal populations in the SCN are vulnerable to EtOH-induced insult during brain development. The observed alterations in SCN neurons containing VIP may have an impact upon clock responses to light input and thus contribute to the long-term effects of neonatal alcohol exposure on the photic regulation of circadian behavior.

Keywords

Suprachiasmatic nucleus; Vasoactive intestinal polypeptide; Neuropeptides; Ethanol; Circadian rhythm; Clock; Entrainment; Fetal alcohol spectrum disorders

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Introduction

In humans, alcohol abuse during pregnancy produces a variety of deleterious effects in exposed offspring, including structural and cellular damage within the central nervous system (CNS). Reductions in brain size and in the volume of specific brain regions such as the anterior cerebellum and corpus callosum provide notable examples of CNS structural defects that occur in children exposed to alcohol in utero (Roebuck et al., 1999). These alcohol-induced changes in CNS structure are often permanent and thus have long-term functional implications in the neural regulation of behavior for exposed individuals. Detailed studies using a rat model similarly indicate that alcohol exposure during the neonatal period, which corresponds to the third trimester equivalent in human brain development (Dobbing and Sands, 1979), produces reductions in brain size and weight (Maier et al., 1996); cell loss in the cerebellum, hippocampus, and olfactory bulb (Bonthius et al., 1992; Bonthius and West, 1990; Chen et al., 1998; Goodlett et al., 1998); altered neuronal circuitry (West et al., 1981); and decreased expression of specific neurochemical signals (Manteuffel, 1996). In the cerebellum and hippocampus, the structural manifestations of neonatal alcohol exposure are, respectively, coupled with chronic deficits in motor performance and learning/memory tasks (Goodlett et al., 1987, 1988; Kelly et al., 1988; Thomas et al., 1996, 1998). Further application of this or similar animal models is necessary to elucidate the scope of the long-term CNS structural damage and dysfunction associated with alcohol exposure during brain development and to advance our understanding beyond known clinical correlates in affected offspring.

Recent studies have revealed that the neural clock responsible for the regulation of circadian or 24-h rhythms is susceptible to alcohol-induced damage during CNS development and corresponding neurobehavioral disturbances (Earnest et al., 2001). In mammals, the suprachiasmatic nucleus (SCN) of the anterior hypothalamus functions as the internal biological clock that governs the generation of circadian rhythms in a variety of biological processes (Moore, 1983). Complementary to its role as the master circadian clock, the SCN mediates the synchronization or entrainment of circadian rhythms to light–dark cycles. The retinohypothalamic tract (RHT), a monosynaptic projection from a subpopulation of retinal ganglion cells, is the primary pathway by which entraining light–dark signals are communicated to the SCN. RHT fibers terminate bilaterally on SCN neurons containing vasoactive intestinal polypeptide (VIP) located within the ventrolateral subfield of the SCN (Ibata et al., 1989; Moore, 1983; Pickard, 1982). Initial evidence for alcohol-induced damage to the RHT–SCN photoentrainment pathway during brain development is derived from functional studies indicating that prenatal exposure to alcohol alters the light–dark regulation of the rat rhythm in deep body temperature (Sei et al., 2003). Consistent with this effect of alcohol exposure during brain development on SCN timekeeping function, our previous studies demonstrate that neonatal alcohol treatment produces long-term changes in the photic regulation of rat circadian rhythm in wheel-running behavior, including the pattern of photoentrainment, rate of re-entrainment to shifted light–dark cycles, and phase-shifting responses to light (Allen et al., 2005a, 2005b; Farnell et al., 2004).

The affected neural substrates responsible for these alcohol-induced disturbances in circadian photoentrainment and phase-shifting responses to light are unknown, but may involve permanent structural or cellular changes in the SCN and/or input pathways by which light signals regulate the clock mechanism. VIP-containing neurons in the SCN are potential targets for the modulatory effects of neonatal alcohol exposure on the photic control of circadian rhythms because (1) these neurons receive direct retinal input via the RHT (Ibata et al., 1989); (2) VIP has been implicated in the phase-resetting of circadian rhythms by light (Albers et al., 1984; Piggins et al., 1995); and (3) VIP promotes neuronal survival during brain development (Brenneman and Eiden, 1986). Consequently, the objective of the present study was to examine the long-term impact of neonatal alcohol exposure on VIP neurons within the

SCN. Experiments were conducted to determine whether neonatal alcohol treatment alters VIP mRNA expression and immunostaining for this neuropeptide within the SCN of adult rats. Because perturbations in circadian rhythms and their photoentrainment have been linked to sleep-wake disturbances, bipolar affective disorder (i.e., manic-depressive illness) and depression (Moore, 1991; Schwartz, 1993), all of which are known neurobehavioral correlates of prenatal alcohol exposure in humans (Sher, 2004), this analysis may yield critical insight into how alcohol exposure during rapid brain growth produces pathologies in the circadian regulation of sleep and other body processes.

Methods

Subjects

The subjects were 70 male Sprague-Dawley rat pups derived from 18 time-mated litters. To maintain consistency with our previous studies analyzing the long-term effects of neonatal alcohol exposure on circadian behavior (Allen et al., 2005a, 2005b; Farnell et al., 2004), male subjects were used exclusively in the present experiments. All 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, using cross-fostering procedures to maintain equivalent litter size by the addition of nonexperimental pups from other litters. These procedures were implemented to prevent increased weight gain among pups from litters of smaller size and thus reduce the potential influence of accelerated somatic growth as a confounding factor for the dependent variables assessed in this study. On PD 4, the rat pups were assigned to one of five treatment groups: a suckle control (SC) group ($n = 14$) and four artificial-rearing treatment groups. To minimize the potential confound of litter effects, no more than two pups from the same litter were assigned to the same treatment condition. The artificial-rearing groups received either alcohol (EtOH) at 3.0 (EtOH3.0; $n = 14$), 4.5 (EtOH4.5; $n = 14$), or 6.0 (EtOH6.0; $n = 14$) g/kg/day or maltose–dextrin (gastrostomy control [GC]; $n = 14$) from PD 4 through 9. All experimental analyses were performed on animals at 4 months of age. Because VIP mRNA expression and peptide immunoreactivity in the rat SCN show diurnal oscillations during exposure to light–dark cycles (Albers et al., 1990; Morin et al., 1991; Takahashi et al., 1989) but no evidence of circadian rhythmicity in DD (Ban et al., 1997; Shinohara et al., 1993), brain tissue was collected at a single timepoint from animals that were exposed to DD beginning at 1800 h (i.e., lights-off in LD 12:12) and sacrificed 18 h later (1200 h or circadian time [CT] 6). Selection of this timepoint for analysis was based on preliminary studies indicating that VIP mRNA levels and VIP-immunoreactive (ir) cells in the SCN are comparably high at CT 6 in SC and GC rats with no significant variation over the rest of the cycle (Nahm et al., 2005). During exposure to DD, experimental procedures were performed using an infrared viewer (FJW Optical Systems, Palatine, IL). The procedures and research in this study were approved by the University Laboratory Animal Care Committee at Texas A&M University and conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals.

Artificial rearing

The artificial-rearing process has been described previously in detail (West, 1993). Briefly, on PD4, pups were anesthetized with isoflurane (VEDCO Inc., St. Joseph, MO) and gastrostomy tubes were inserted down the esophagus and surgically implanted into the stomach (Diaz, 1991; West et al., 1984). From PDs 4 to 9, gastrostomized 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). Gastrostomized pups received either alcohol treatment or isocaloric maltose–dextrin

solution from PDs 4 to 9. On PD 10, artificially reared pups were fostered to a lactating dam after their gastrostomy tubes had been cut and sealed. SC animals were maintained with lactating dams and were included to control for any effect produced by artificial-rearing methods. Identification of subjects from different treatment groups was accomplished by subcutaneous implantation of each pup with a coded microchip (Avid, Los Angeles, CA) on PD 10. Animals were weaned on PD 21 and housed two to three per cage. After weaning, food and water were provided ad libitum for the remainder of the experiment.

Drug administration

Beginning around midday of the LD 12:12 (1200 h) from PD 4 to 9, alcohol solutions of 6.8%, 10.2%, or 13.6% (vol/vol) were administered to the EtOH groups through 2 of the daily 12 feedings to yield alcohol doses of 3.0, 4.5, or 6.0 g/kg/day, respectively. These doses of alcohol and binge-like treatment regimen have been shown to consistently produce dose-related, long-term neuroanatomical damage (Bonthius et al., 1992; Bonthius and West, 1988, 1990; Chen et al., 1998; Chen and West, 1999; Livy et al., 2003). The GC group (0 g/kg/day alcohol) was given the appropriate concentration of isocaloric maltose–dextrin solution in place of alcohol. All animals received regular formula diet during the 10 remaining daily feedings.

Blood alcohol concentration

Blood alcohol concentration (BAC) for all EtOH-treated pups was measured using a gas chromatograph (Model# 3900, Varian, Palo Alto, CA). Blood samples (20 μL) were collected from pup tails 90 min after the beginning of the second alcohol feeding on PD 6 (peak BAC) (Bonthius et al., 1988; Kelly et al., 1987). Samples were stored in glass vials containing a 200-μL cocktail composed of 0.6-M perchloric acid and 4-mM *n*-propanol in double distilled water until the initiation of BAC analysis.

Immunocytochemistry

For immunocytochemical localization of VIP, anesthetized animals (sodium pentobarbital; 100 mg/kg) were sacrificed by transcardiac perfusion with 100 mL of 0.1-M phosphate buffer (pH = 7.4) containing heparin (2 U/μL) followed by 250 mL of 4% paraformaldehyde. Immediately after perfusion, the brains were removed, post-fixed overnight at 4°C, and then stored for at least 24 h in cryoprotectant solution (30% sucrose in 0.15-M phosphate buffer) prior to sectioning. The tissue was then frozen and sectioned serially in the coronal plane at 30 μm using a sliding microtome.

Immunostaining procedures for the localization of VIP were conducted on brain sections throughout the rostral-caudal extent of the SCN. VIP immunocytochemistry in this study was performed on four different sets of brain sections with each set containing sections from equal numbers of SC, GC, and EtOH animals. VIP immunostaining was accomplished using a modified avidin–biotin–immunoperoxidase method. With interceding washes, sections were sequentially incubated in a solution of 10% normal donkey serum in 0.15-M phosphate buffer for 20 min, in rabbit anti-VIP (Peninsula Laboratories, San Carlos, CA) diluted 1:3,000 in buffer containing 1% blocking serum, 0.3% triton, and 0.25% BSA for 48 h; in biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch, WestGrove, PA) diluted 1:1,000 in 0.15 M phosphate buffer for 2 h; and in avidin–biotin–peroxidase complex (Elite-ABC kit; Vector Labs, Burlingame, CA) for 2 h. Tissue sections were repeatedly washed in 175-mM sodium acetate–10-mM imidazole buffer and then reacted for 7–10 min with a chromogen solution consisting of 0.03% DAB, 0.1 M $NiSO_4$-$7H_2O$, and 0.003% hydrogen peroxide in 125-mM sodium acetate–10-mM imidazole buffer (pH = .0). The chromogen reaction was similarly timed across the four different sets of brain sections, so its termination occurred near the end of the linear phase of color development. VIP immunostaining was evident as a blue-black reaction product in the cytoplasm of perikarya and in fibers and terminals. The specificity of

immunostaining for VIP was verified using standard procedural and preabsorption controls. No immunoreactivity was observed in sections subjected to staining procedures in which the primary antisera was omitted or was preincubated with homologous antigen (10^{-5} M).

Stereological methods

The general stereological methods used in the present study were similar to those described previously (Bonthius et al., 1992; Edwards et al., 2002; Smith et al., 2008) with exception that frozen sections were used for cell counting in this investigation. In SC, GC, and EtOH animals ($N = 6$ for all groups), the density of VIP-ir neurons in the SCN was estimated using the optical disector method (Gundersen et al., 1988; West and Gundersen, 1990) and the reference volume (*V*ref) of the SCN was determined by point counting and application of Cavalieri's Principle (Gundersen et al., 1988). Measurements of the *V*ref [$\sum pi \times A(pi) \times t$; where $\sum pi$ is the sum of the number of points (pi) counted, $A(pi)$ is the known area associated with each point, and t is the known distance between two serial sections] and Nv [$\sum Q / \sum$disector $\times A$(fr) $\times h$; where $\sum Q$ is the sum of the VIP-ir neurons counted from each disector frame, $\sum$disector is the sum of the number of disector frames counted, A(fr) is the known area associated with each disector frame, and h is the known distance between two disector planes] were used to estimate the total number (*V*ref × *Nv*) of VIP-ir neurons in the SCN. The C.A.S.T.-GRID software (Olympus Denmark A/S, Albertslund, Denmark) was used for estimating SCN volume and the density of VIP-ir neurons in the SCN. The microscope was equipped with a computer-controlled stage (*X* and *Y* axes), and the attached microcator (Model MT12, Heidehan, Traument, Germany) measured the distance between the two disector planes on the *Z* axis, *h*, which was 10 μm in this study. Following immunocytochemical procedures, the actual thickness of the sections in the *Z* axis was not measured. Instead, the presumed section thickness (30 μm) was used in estimating the *V*ref. The images were transferred to a personal computer (Micron Millennium, Boise, ID) via a Jai color video camera (Model 2040, Copenhagen, Denmark). SCN volume determinations were established using a 10× objective lens and measurements of the density of VIP-ir neurons in the SCN were instituted using a 60× oil-immersion objective lens with a 1.40 aperture.

Statistical analysis was performed using a one-way ANOVA with alcohol treatment as the sole between-subject factor to determine whether the number or density of VIP-ir neurons in the SCN were significantly different among treatment groups, and the Newman–Keuls sequential range test was used post hoc to analyze differences between control- and different EtOH-treated groups if the main effect of alcohol treatment was obtained. The α value was set at 0.05 for all statistical analyses.

Quantitative real-time polymerase chain reaction (PCR) analysis

For analysis of VIP mRNA, SC, GC, and EtOH (3.0, 4.5, and 6.0 g/kg/day) animals ($N = 5$ for all groups) were sacrificed by decapitation and the SCN was immediately dissected as described previously (Earnest and Sladek, 1986; Liang et al., 1998). After the entire brain was removed and sectioned in the coronal plane at the midlevel of the optic chiasm (600–800 μm), the SCN was obtained by dissecting a small square of tissue in ventral extent of the brain section adjacent to the third ventricle. All tissue samples were immediately frozen in liquid nitrogen and stored at −80°C.

Total cellular RNA was later extracted from individual samples using RNeasy Lipid Kit protocols (Qiagen, Valencia, CA) and subjected to on-column DNase-I digestion. Relative quantification of VIP mRNA abundance was performed using SYBR-Green quantitative (qt)-PCR technology (Applied Bio-systems, Inc. [ABI], Foster City, CA) and methods similar to those described previously (Allen et al., 2004a). Total RNA (1 μg) from individual samples was reverse transcribed using random hexamers and Superscript II reverse transcriptase

(Invitrogen, Carlsbad, CA). For each sample, the cDNA equivalent of 20–30 ng of total RNA was amplified in an ABI PRISM 7700 sequence detection system (Heid et al., 1996). This analysis was conducted concurrently on triplicate aliquots of each sample. To control for differences in sample RNA content, cyclophilin A (*CypA*) mRNA was amplified with the cDNA equivalent of 1 ng total RNA from the same samples. The following primers were designed with Primer Express software (ABI): *VIP* forward: 5′-TGCCTTAGCGGAGAATGACA-3′; *VIP* reverse: 5′-CGCTGGTGAAAACTCCATCA-3′; *CypA* forward: 5′-TGTGCCAGGGTGGTGACTT-3′; *CypA* reverse: 5′-TCAAATTTCTCTCCGTAGATGGACTT-3′.

Using the comparative C_T method described in the ABI Prism 7700 Sequence Detection System User Bulletin #2 (PE-ABI), the relative abundance of VIP mRNA was calculated by normalization first to corresponding *CypA* mRNA levels in each sample and then to a calibrator consisting of pooled cDNA from multiple samples that was analyzed on each reaction plate. Relative levels of VIP mRNA were represented as a percentage of the maximal value obtained within an individual experiment.

In situ hybridization

Anesthetized animals (sodium pentobarbital; 100 mg/kg) were sacrificed by transcardiac perfusion with 100 mL of 0.1-M phosphate buffer (pH = 7.4) containing heparin (2 U/μL) followed by 250 mL of 4% paraformaldehyde. The brains were immediately removed, placed in M-1 embedding matrix (Thermo Fisher Scientific, Waltham, MA), and frozen in an ethanol bath. The tissue was later sectioned in the coronal plane at 10 μm using a cryostat and sections were mounted on charged slides. In situ hybridization analysis for VIP mRNA was conducted using three oligonucleotide probes that encode specific 29-, 40- and 42-base sequences encompassing nucleotides 521–549, 173–212, and 413–454 of the rat VIP cDNA. Specific hybridization of these probes to VIP mRNA has been demonstrated previously in the rodent brain (Ban et al., 1997; Trembleau et al., 1993). Labeling of these oligonucleotide probes with digoxigenin-dUTP was performed using the DIG Oligonucleotide Tailing Kit (Roche Applied Science). The following procedures were sequentially performed on mounted sections: (1) thaw at room temperature for 30 min; (2) treat with proteinase K (10 μg/mL) at room temperature for 10 min; (3) dehydrate and rehydrate in ethanol grades; (4) rinse in 2X SSC (0.15 M NaCl, 0.015 sodium citrate, pH = 7.2); (5) incubate in prehybridization buffer containing 50% formamide, 4X SSC, 1X Denhart's solution, 10% dextran sulfate, 0.01 M dithiothreitol, 0.25 μg/mL tRNA, and 2 mg/mL sarkosyl for 30 min at 37°C; (6) hybridize overnight at 37°C with labeled oligonucleotide in hybridization buffer (600 μL/section); (7) rinse in 2X SSC, for 30 min in 0.2X SSC and for 20 min in 1X SSC at room temperature; (8) incubate for 30 min in blocking reagent (Roche Applied Science, Indianapolis, IN) and then for 3 h at room temperature with polyclonal sheep anti-digoxigenin Fab fragments conjugated to horse-radish peroxidase diluted 1:15 in blocking solution; (9) wash in 125-mM sodium acetate–10-mM imidazole buffer (pH = 7.0); (10) incubate for 10 min with a chromogen solution consisting of 0.03% DAB, 0.1 M Ni-SO_4-$7H_2O$, and 0.003% hydrogen peroxide; and (11) rinse in 0.15-M phosphate buffer to terminate the reaction. Slides were dehydrated through alcohol grades, cleared in Histoclear and coverslipped with Permount®. Hybridization signal was evident as a blue-black reaction product within the cytoplasm of perikarya, but was absent in brain sections incubated with noncomplementary (sense) probe for VIP mRNA.

Results

Blood alcohol concentration

Administration of alcohol at doses of 3.0, 4.5, or 6.0 g/kg/day produced peak BACs (mean ± standard error of the mean) on PD 6 of 131.1 ± 1.1 mg/dL (0.131%), 244.0 ± 9.3 mg/dL

(0.244%), and 323.7 ± 19.8 mg/dL (0.323%), respectively. These BAC values are comparable to those established in previous studies using similar doses of alcohol (Bonthius and West, 1988, 1990; Napper and West, 1995). The BACs are noteworthy because the values established with the low dose of alcohol (3.0 g/kg/day) were within the range of those achieved by social drinkers, and the levels produced by higher doses are often sustained by individuals who abuse alcohol (Urso et al., 1981).

Effects of neonatal alcohol exposure on VIP mRNA expression in the SCN

The long-term effects of neonatal alcohol exposure on VIP expression in the SCN were first examined using qt-PCR methods to gauge treatment-induced changes in mRNA abundance. SC-, GC-, and EtOH-treated animals exhibited comparable levels of VIP mRNA in the SCN with no significant differences between treatment groups (Fig. 1). In situ hybridization analysis similarly revealed that neonatal alcohol treatment had no discernible impact on the anatomical distribution of VIP mRNA-expressing cells in the SCN. In all control- and EtOH-treated animals, cells exhibiting hybridization signal for VIP mRNA were evident throughout the SCN with exception of its rostral and caudal poles and were heavily clustered within the ventrolateral subfield of the nucleus immediately adjacent to the optic chiasm (Fig. 2). Hybridization signal was not observed in adjacent regions of the anterior hypothalamus.

Effects of neonatal alcohol exposure on VIP-ir neurons in the SCN

In conjunction with the analysis of VIP mRNA expression in the SCN, immunocytochemical methods were used to examine the long-term effects of neonatal alcohol treatment on VIP-containing neurons in the SCN. VIP immunoreactivity was distinguishable within the cytoplasm of small SCN neurons and within fibers ramifying locally especially in the dorsal portion of the nucleus (Fig. 3). Consistent with the observed distribution of VIP mRNA, immunopositive perikarya were located in the middle two-thirds of the SCN along its rostral-caudal axis and concentrated within the ventrolateral subdivision. Stereological determinations of SCN *V*ref and the density and number of SCN neurons expressing VIP immunoreactivity in the SC, GC, and EtOH groups are depicted in Fig. 4. Importantly, the observed numbers of VIP-ir neurons in the SCN of both control groups were comparable to those reported for untreated adult rats in previous studies (Chee et al., 1988;Madeira et al., 1997). One-way ANOVAs comparing each of the dependent measures among all five treatment groups indicated that SCN *V*ref was not significantly different between treatment groups. However, similar analysis of the data for the density and number of VIP-ir neurons within the SCN revealed a main effect of treatment on these measures ($F[4, 35] = 6.68$ and $F[4, 35] = 9.88$, respectively; $P < .0005$). Based on post hoc analyses, the density and number of SCN neurons with VIP immunoreactivity in rats treated with 6.0 g/kg/day EtOH were significantly decreased ($P < .001$) in comparison with the values found in both control groups. At this dose, neonatal alcohol exposure induced a 35–40% decrease in the density and number of VIP neurons in the SCN of adult rats (Fig. 4). Furthermore, these measures were significantly lower ($P < .05$) in all three EtOH-treated groups than in SC animals.

Discussion

The present results demonstrate that alcohol exposure during the brain growth spurt has long-term effects on VIP-containing neurons in the SCN. At the highest dose, neonatal alcohol treatment produced substantial decreases in the number and density of VIP-ir neurons with no corresponding sign of altered mRNA expression in the SCN of adult rats. Reductions in the total number of SCN neurons containing VIP have been similarly observed in response to chronic alcohol treatment for 6 months (Madeira et al., 1997). Based on previous evidence indicating that neonatal alcohol exposure has little or no impact on SCN neuronal number and density in adult rats (Farnell et al., 2004), it seems unlikely that the observed decreases in the

stereological profile of VIP neuronal populations are a consequence of alcohol-induced cell loss in the SCN. Instead, the reduction in VIP neuronal number and density may reflect alcohol-related changes in the production of this peptide by SCN neurons. An alternative explanation for the present findings is that neonatal alcohol exposure may induce a permanent shift in the intracellular distribution of VIP within SCN neurons. In the rat, most of the 8,000–10,000 neurons in each SCN are distinguished by parvicellular morphologies (van den Pol, 1980) in which neuropeptides, such as VIP, are transported and differentially stored in fibers and terminals after their synthesis in the cell body. Thus, the decreased number and density of VIP-ir perikarya in the SCN of EtOH-treated rats may be indicative of a further shift toward axonal storage rather than reduced production of this neuropeptide. In this regard, animals receiving even the highest EtOH dose exhibited no perceptible increase in the distribution VIP immunoreactivity within fibers. However, the present findings provide limited opportunity to distinguish between these explanations for the observed decrease in SCN neurons containing VIP because (1) immunocytochemistry is not an optimal method for quantifying differences in protein levels and (2) analysis of SCN production of this peptide using quantitative methods and of changes in axonal peptide storage is complicated due to the fiber projections of VIP-containing neurons in the SCN to other brain regions.

These decreases in the number and density of VIP-containing neurons in the SCN may have important implications in the effects of alcohol exposure during the brain growth spurt on the photic entrainment and phase-shifting responses of circadian rhythms. Neonatal alcohol treatment produces long-term perturbations in the photic regulation of the circadian rhythm in rat wheel-running activity, including the phase angle of entrainment to LD 12:12 (Allen et al., 2005b), the rate of re-entrainment to an abrupt shift in the light–dark cycle (Allen et al., 2005a), and phase-shifting responses to brief pulses of light (Allen et al., 2005a; Farnell et al., 2004). The potential link between the observed reduction in the population of VIP neurons within the SCN and these alterations in the light–dark regulation of the activity rhythm in alcohol-exposed rats is corroborated by the observations that (1) SCN neurons containing VIP receive direct retinal innervation (Ibata et al., 1989); (2) when delivered to the SCN in vivo or in vitro, this neuropeptide produces phase shifts similar to those induced by light (Albers et al., 1984; Meyer-Spasche and Piggins, 2004; Piggins et al., 1995); and (3) circadian entrainment to LD cycles and phase-shifting responses to light pulses are altered in mice lacking the VIP receptor, VPAC2 ($Vipr2^{-/-}$) (Hughes and Piggins, 2008).

Another possibility is that the diminished population of VIP neurons in the SCN may impact upon local intercellular communication and the effects of neonatal alcohol exposure on this process may contribute to the changes in the photoentrainment, light-induced phase shifts, and even the free-running period of the activity rhythm in adult rats. The SCN contains a population of cell-autonomous clocks and coordination of circadian timekeeping among multiple cellular clocks is critical for its ensemble function as a pacemaker that regulates rhythmicity in other cells and tissues. VIP is thought to function as a key signal in the intercellular coupling and synchronization among SCN clock cells because (1) axon collaterals of many VIP neurons in the SCN terminate locally and provide a basis for widespread intranuclear communication (Moore, 1983; van den Pol and Tsujimoto, 1985) and (2) VIP-deficient mice are distinguished by the disruption of circadian rhythms in constant conditions (Colwell et al., 2003). Thus, the alcohol-induced decrease in SCN neurons containing VIP may influence the coordination of rhythmicity among individual cellular clocks. In turn, disturbances in the VIP-mediated modulation of oscillator coupling in the SCN may play a role in the reported alterations in free-running period, pattern of photoentrainment, and phase-shifting responses to light because modeling based on research findings predicts that these fundamental properties of overt circadian rhythms are dependent on the strength of the coupling between multiple autonomous oscillators comprising the central pacemaker (Pittendrigh and Daan, 1976).

Collective evidence from this and other studies indicates that alcohol exposure during brain development permanently alters cellular and molecular components of the SCN circadian clock as well as its regulation of overt behavioral and physiological rhythms. Alcohol treatment during the prenatal or neonatal period has been previously shown to produce long-term changes in (1) the temporal configuration of essential "gears" in the molecular clockworks (Farnell et al., 2008); (2) endogenous rhythmicity in the SCN (Allen et al., 2004b); (3) circadian rhythms of core body temperature and plasma corticosterone levels (Handa et al., 2007); (4) fundamental circadian properties of the activity rhythm, including the pattern of entrainment to light–dark cycles, phase-shifting responses to light, and its free-running period (Allen et al., 2005b; Farnell et al., 2004); and (5) rate of re-entrainment to shifted light–dark cycles (Allen et al., 2005a; Sei et al., 2003). These permanent alcohol-induced alterations in the regulation of circadian behavior appear to mirror some of the neurobehavioral disturbances in fetal alcohol syndrome (FAS) and fetal alcohol spectrum disorders (FASDs) (Chudley et al., 2005; Hoyme et al., 2005). Perturbations in circadian rhythms like those induced by alcohol exposure during brain development are thought to play some role in sleep-wake and affective disorders (Moore, 1991; Schwartz, 1993), both of which are known clinical correlates of FAS (Sher, 2004). In this regard, it is interesting to note that the fragmented activity patterns and altered photoentrainment of EtOH-treated rats in our studies are analogous to clinical sleep-wake disturbances observed in human neonates, children, and adolescents following prenatal exposure to alcohol (Rosett et al., 1979; Smith and Eckardt, 1991; Steinhausen and Spohr, 1998; Steinhausen et al., 1993). Because decreases in the number of VIP neurons within the SCN are coupled with changes in human circadian behavior in patients with Alzheimer's disease (Satlin et al., 1995; Zhou et al., 1995), similar effects of alcohol on VIP neurons within the SCN and the circadian regulation of behavior may contribute to the manifestation of sleep-wake disturbances and even affective disorders in FAS and FASD. Thus, further analysis is necessary to determine whether alcohol exposure in utero produces other human health problems that have been associated with circadian rhythm pathologies such as cancer, cardiovascular accidents, obesity, and diabetes.

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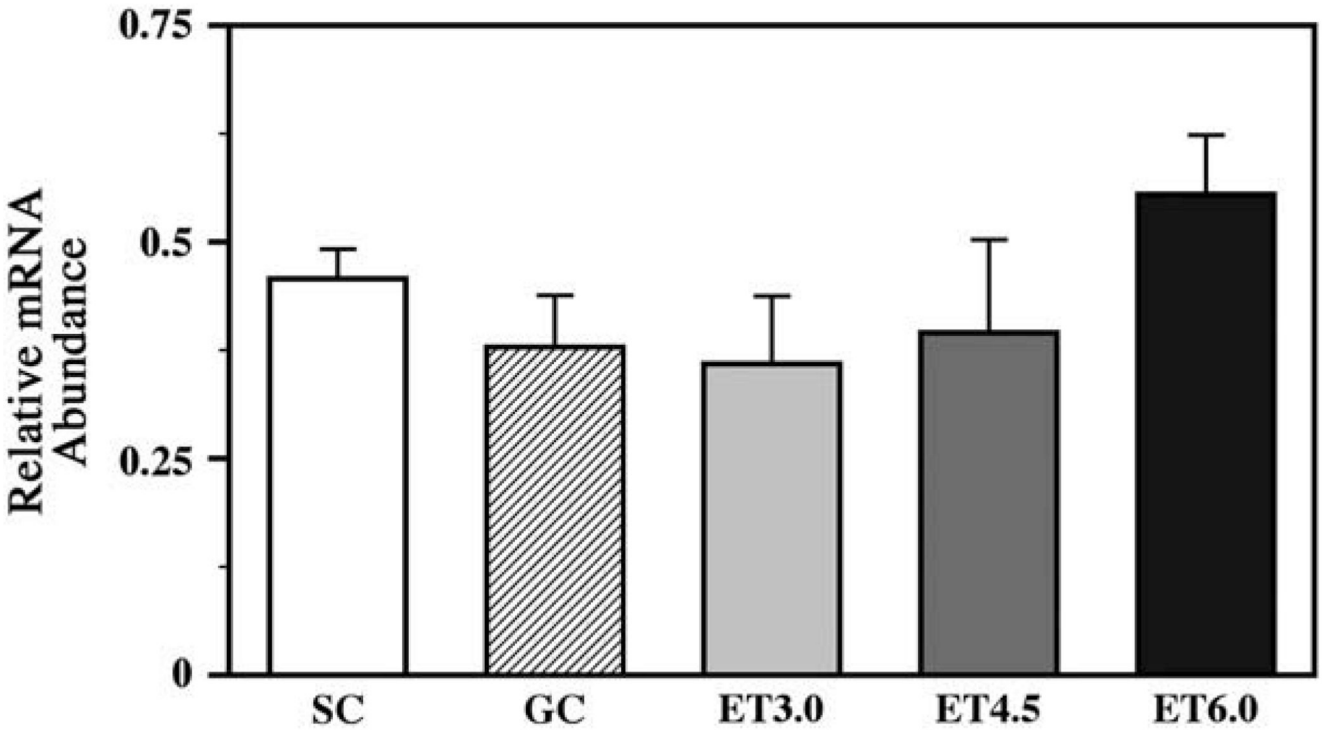

Fig. 1.

Relative abundance of vasoactive intestinal polypeptide (VIP) mRNA in the suprachiasmatic nucleus (SCN) of suckle control- (SC), gastrostomy control- (GC), and EtOH-treated (EtOH 3.0, 4.5, and 6.0 g/kg/day) rats. Bars denote quantitative PCR determinations of *VIP* mRNA levels (mean ± standard error of the mean) in SCN tissue collected from 8 to 12 animals. The plotted values for the relative mRNA abundance correspond to the ratios of species-specific *VIP*/*CypA* mRNA signal. EtOH treatment had no significant effects on SCN levels of *VIP* mRNA.

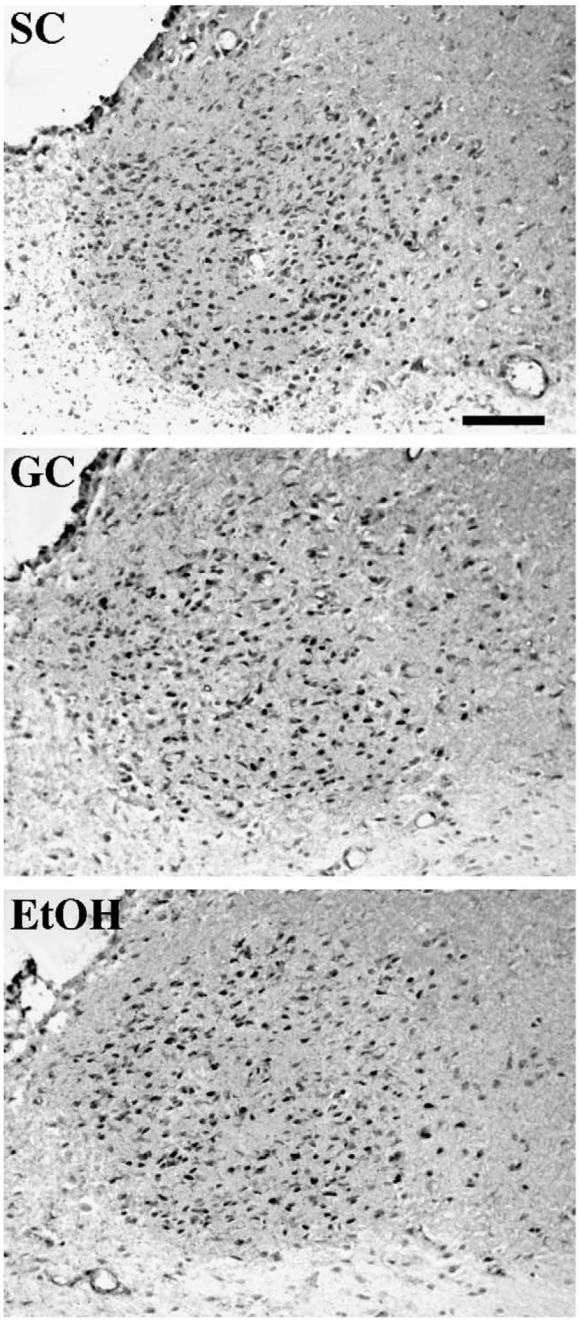

Fig. 2.

Representative brightfield photomicrographs of the rat suprachiasmatic nucleus (SCN)-optic chiasm region illustrating the anatomical localization of vasoactive intestinal polypeptide (VIP) mRNA-expressing cells in suckle control (SC), gastrostomy control (GC), and EtOH 6.0 rats. In all treatment groups, perikarya with nonisotopic hybridization signal for VIP mRNA are similarly distributed within the ventral SCN. Scale bar = 100 μm.

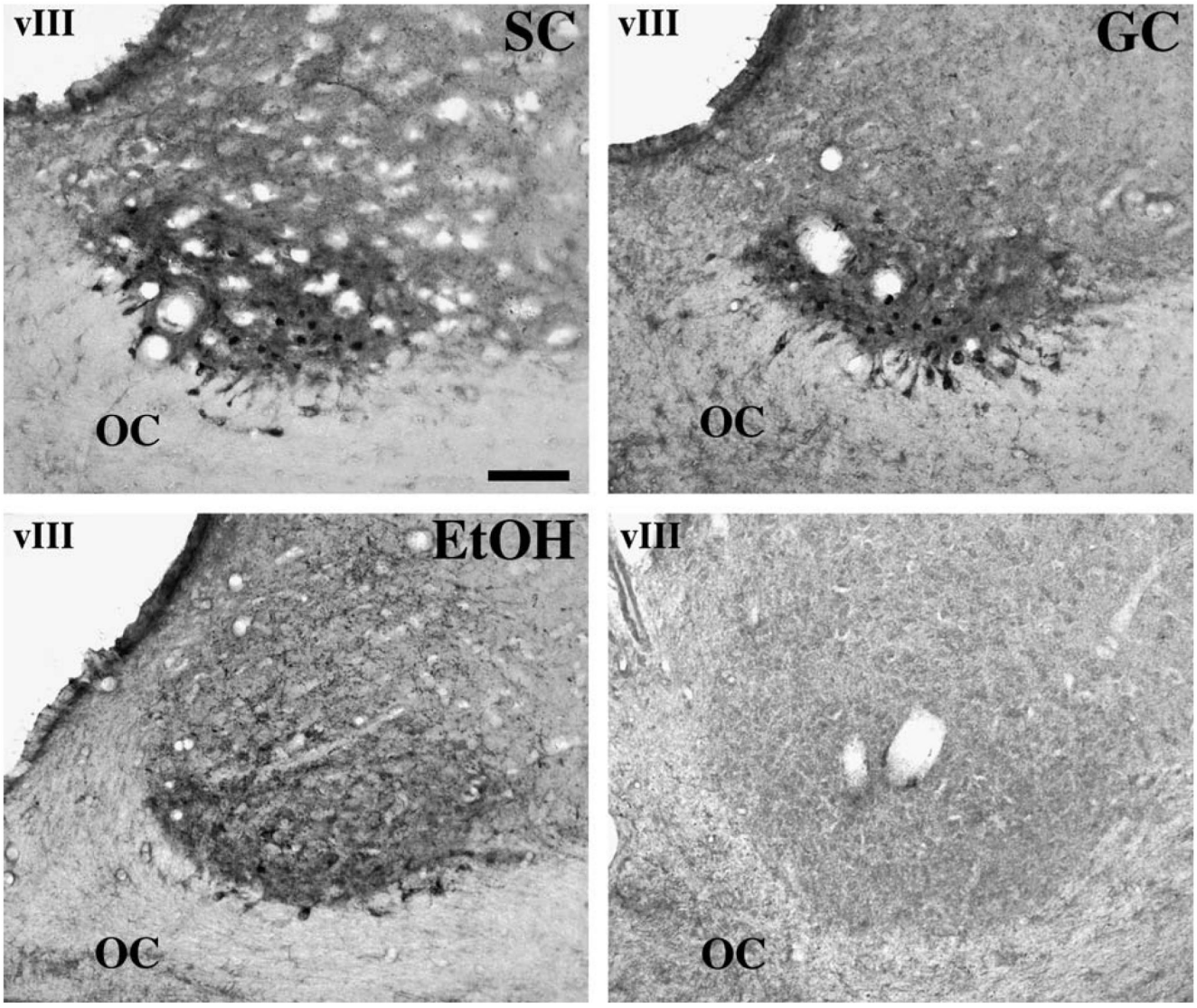

Fig. 3.

Representative brightfield micrographs of the rat suprachiasmatic nucleus (SCN)-optic chiasm region depicting the cellular distribution of vasoactive intestinal polypeptide (VIP) immunoreactivity in suckle control (SC), gastrostomy control (GC), and EtOH 6.0 rats. VIP immunostaining was localized within fibers and scattered perikarya in ventral SCN. The bottom, right panel represents a control section that was subjected to staining procedures in which the VIP antisera was preincubated with homologous antigen (10^{-5} M). OC, optic chiasm; vIII, third ventricle. Scale bar = 100 μm.

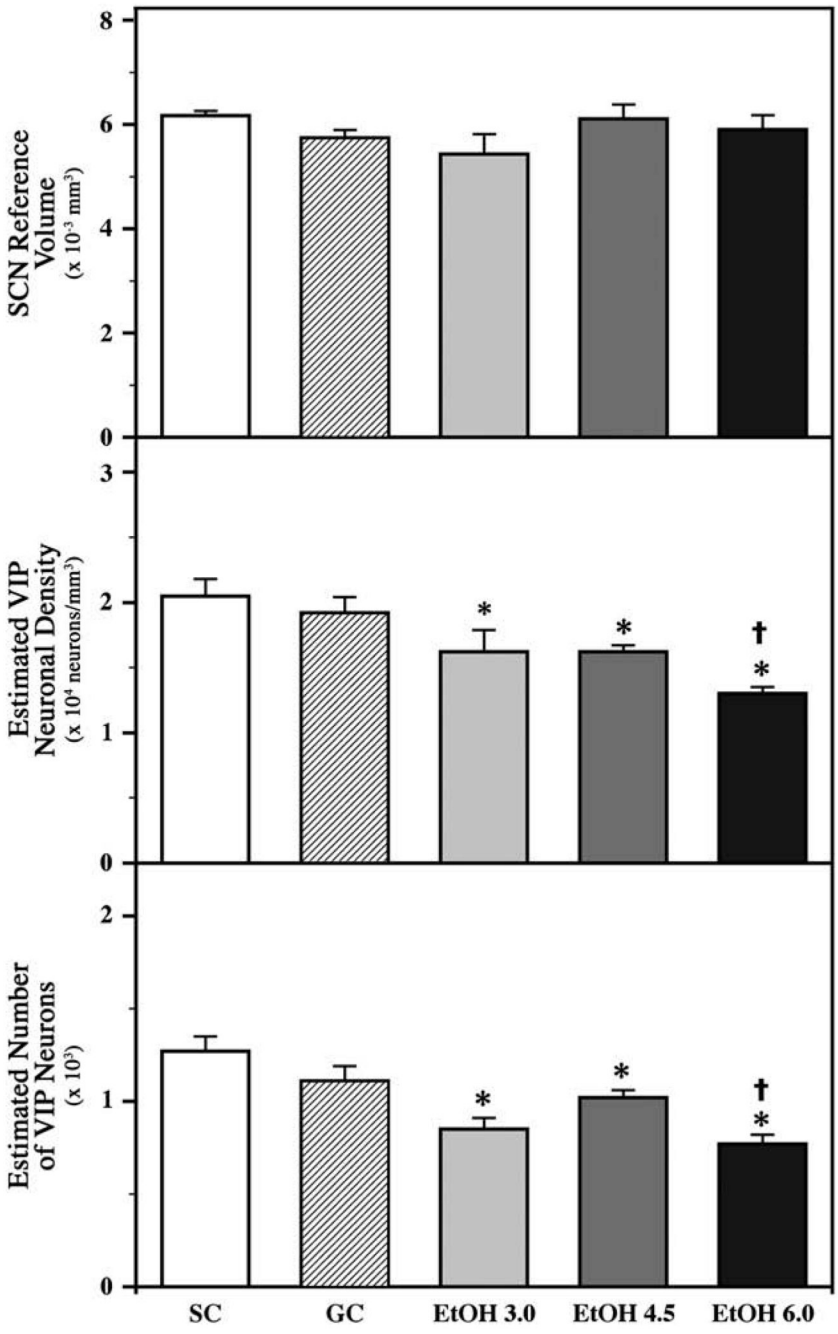

Fig. 4.

Effects of neonatal alcohol exposure on the reference volume, density, and estimated number of vasoactive intestinal polypeptide (VIP)-ir neurons in the suprachiasmatic nucleus (SCN) of suckle control- (SC), gastrostomy control- (GC), and EtOH-treated (EtOH 3.0, 4.5, and 6.0 g/kg/day) rats. Bars represent mean (±standard error of the mean) values. The density and number of VIP-ir neurons in the SCN of rats treated with 6.0 g/kg/day EtOH were significantly decreased (†P <.001) in comparison with the values found in both control groups. These measures were significantly lower (*P <.05) in all three EtOH-treated groups than in SC animals.