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Perinatal alcohol exposure leads to prolonged upregulation of hypothalamic $GABA_A$ receptors and increases behavioral sensitivity to gaboxadol

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Abstract

Prenatal alcohol exposure (AE) is associated with lasting abnormalities of sleep and motor development, but the underlying mechanisms are unknown. We hypothesized that AE alters development of GABAergic signaling in the hypothalamic regions important for the control of sleep and motor activity. Alcohol (5.25 g/kg/day) was administered intragastrically to male rats on postnatal days (PD) 4–9, a period of brain development equivalent to the human third trimester (AE group). Control pups were sham-intubated (S group). Motor activity was monitored on PD27–28. On PD29–30, $GABA_A$ receptor subunit mRNA levels and α4 and δ subunit proteins were quantified by RT-PCR and immunoblotting, respectively, in the wake- and motor activity-promoting perifornical (PF) region of the posterior hypothalamus and the sleep-promoting ventrolateral preoptic (VLPO) region of the anterior hypothalamus. Then, in 47–52-day old rats, motor activity was quantified following administration of $GABA_A$ receptor agonist, gaboxadol (5 mg/kg s.c.). In the PF region, mRNA and protein levels for the α4 and δ subunits were significantly higher and β3 and γ2 subunit mRNAs were also increased in the AE group. In the VLPO region, only the δ subunit mRNA was increased. Spontaneous motor activity was lower and suppressed more by gaboxadol in the AE than S group, and the latency to a transient total loss of activity after gaboxadol was shorter in the AE group. Thus, perinatal AE leads to $GABA_A$ receptor overexpression in the vigilance- and motor activity-promoting hypothalamic PF region, with the neurochemical and functional outcomes lasting long beyond the period of the insult.

Keywords

development; ethanol; GABA; hypothalamus; motor activity; sleep

Introduction

Alcohol (ethanol) consumption during pregnancy has grave and multifaceted consequences for child development, such as the fetal alcohol syndrome and related neurodevelopmental disorders collectively referred to as the fetal alcohol spectrum disorders (FASD) (reviewed in [25]). Among those, abnormal sleep patterns that correlate with impairments of motor and

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cognitive development during the first months of life have been observed in infants [7,13,15]. When tested in animal models, sleep abnormalities can persist through adulthood [8,33,35], but the neurochemical mechanisms underlying the disrupted development and control of sleep caused by prenatal alcohol exposure (AE) have not been elucidated.

One well documented target of AE in the developing brain is the signaling mediated by gamma-aminobutyric acid (GABA) type A receptors ($GABA_AR$) (e.g., [2,4,14,29,31]). $GABA_ARs$ are pentamers that are assembled from at least 19 subunits known to date (reviewed in [30]). Different combinations of subunits result in functional receptors with different pharmacology, including responses to alcohol [30,34,40].Importantly, $GABA_ARs$ also play a key role in the regulation of sleep. Sleep-promoting GABAergic neurons are clustered within the ventrolateral preoptic area (VLPO) of the anterior hypothalamus and innervate multiple wake-promoting brain sites including the perifornical (PF) region of the posterior hypothalamus that has wake- and motor activity enhancing functions [19,22,32,36]. Activation of $GABA_ARs$ in the PF region promotessleep [1,27]. In rats, hypothalamic mechanisms start playing a major role in the consolidation of sleep between postnatal day (PD) 2 and 8 [17], and hypothalamic GABA concentrations are dramatically increased between PD 5 and 10 [11]. This period of brain growth spurt in rats is equivalent to the period of human brain development during the third trimester of pregnancy [9]. The findings that neurons of the hypothalamic circadian clock are strongly affected in a rat model of AE at this age [3,10] also suggest that the hypothalamic regulation of sleep and motor activity is vulnerable to alcohol during this period. We hypothesized that exposure to alcohol during the brain growth spurt equivalent to the third trimester of human gestation may lead to a long-lasting upregulation of $GABA_A$ receptor function in the hypothalamic sleep- and motor activity controlling sites, and that this may be associated with a long-lasting increase in sensitivity to sedative and hypnotic effects of GABA.

Materials and methods

We used an established rat model of prenatal AE and followed the protocol described previously in which alcohol is administered to neonatal rats via intragastric intubations [12, 16,21,39]. The procedures for animal handling followed the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Neonatal treatments were administered to male, Sprague-Dawley rats. Seven litters from timed-pregnant rats were culled and cross-fostered among lactating dams to bring each litter size to 8–10 pups. On PD4, the pups within each litter were randomly assigned to either alcohol- (AE) or sham-treated (S) group. The AE group received a daily dose of 5.25 g/kg of alcohol on PD4 through 9 administered as two intragastric intubations per day (2.625 g/kg per intubation, 11.9% v/v in a custom milk formula, 2 h apart). Data indicate that such an exposure results in cognitive deficits in 30 day-old rats 5 [39]. Two hours after the second intubation, an additional intubation with milk only was administered to the AE group to offset reduced maternal milk consumption. The S group underwent the same daily routine of intubations, but no fluid was infused [12,16,39]. Pups were weighed daily (Fig. 1A) and stayed with the dam until weaned on PD21–22. Blood was collected on PD4 from a separate group of animals (n=7) sacrificed 2 h after the second alcohol intubation, and blood alcohol concentration (BAC) was determined using NAD-ADH reagent (Sigma, USA).

On PD29–30, 500 μm-thick hypothalamic slices were obtained from rats sacrificed at a constant circadian time (2–4 pm). Two pairs of tissue micropunches, 700 μm in diameter, were cut bilaterally, one from the PF region of the posterior hypothalamic slice (Fig. 1B) and the other from the VLPO region of the anterior hypothalamic slice. One of the samples from each pair was subjected to RNA extraction and quantitative reverse transcription-polymerase chain

reaction (RT-PCR) using the protocol described in our earlier study [38]. We quantified mRNA levels for eleven subunits of $GABA_AR$ (α1–5, β1–3, γ2 (long and short splicing variants; $\gamma2_{L/S}$), δ, and ε) that are expressed in the hypothalamus [24]. The other sample was frozen on dry ice and stored at −80°C for protein immunodetection. Each slice was then fixed in formalin and cut into 30 μm thick sections that were subsequently stained with Neutral red to verify the micropunch location (Fig. 1B).

$GABA_AR$ subunit-like protein levels for α4 or δ subunits were quantified using dot-blot immunodetection [43]. The starting amount of tissue required for the assay is much smaller than in any other immunoblotting technique, which allowed us to quantify both mRNA and protein in symmetrically located tissue micropunches of the same size. Samples were solubilized and 0.5 μl of the crude extract was absorbed onto a nitrocellulose membrane. All samples from both groups were placed together on one membrane, and then sequentially incubated in primary antibodies against either α4 (Affinity BioReagents, USA; OPA1-04102, 1:1,000) or δ subunit (Chemicon, USA; AB9752, 1:1,000), biotinylated secondary antibodies, avidin-horseradish peroxidase complex (ABC kit, Vector, USA), and then visualized with diaminobenzidine with nickel ammonium sulphate. The images of membranes were digitized and immunostaining density was quantified using ImageJ software (National Institutes of Health, http://rsb.info.nih.gov). Quality controls included histological verification of punch locations and assessment of RNA and PCR quality, as described previously [38].

On PD27–28 and then on PD47, basal motor activity was monitored during the lights-on (rest) period (11:00 am – 2:00 pm) using a system of infrared light beams (AccuScan Instruments, USA). During each session, two rats, one from the AE and one from S group, were tested simultaneously in their separate home cages. Each recording session lasted 90 min, with the number of separate movements averaged over successive 30-min intervals using the software supplied by the manufacturer. On PD51–52, using the same recording setup, we assessed the effect of $GABA_A$ agonist, gaboxadol, whose effects are mediated by α4- and δ subunit-containing $GABA_ARs$ [5,26,41]. Animals from both groups were tested in pairs following s.c. administration of gaboxadol (THIP, Sigma; 5 mg/kg [20]) dissolved in isotonic saline at the beginning of the recording session [5].

The significance of differences between the AE and S groups was examined using one-way ANOVA with Bonferroni's correction (Analyse-It, UK). The differences were considered significant when $P<0.05$.

Results and discussion

Mean BAC in AE rats was 351±24 (SE) mg/dl, consistent with previously published results obtained from a similar experimental design [14,16,21]. Body weight gains were significantly lower in pups of the AE than S group on PD6 through 16 (Fig. 1A) reflecting a growth lag similar to that described in earlier studies [16,18,21,39]. This difference was not significant on PD29–30 (100±4 (SE) g in AE vs. 107±3 in S, $p=0.16$, $n=12$). The difference in body weight gain during the treatment was probably caused by reduced caloric intake due to inhibited suckling in AE rats because feeding with a high-caloric fat emulsion instead of balanced milk formula eliminates this difference [18]. Since we used a mixed litter design, we cannot exclude a possibility of growth disadvantage of AE pups resulting from a selectively altered maternal interaction.

The integrated density of $GABA_AR$ subunit immunostaining was positively correlated with mRNA levels measured in symmetrically located samples obtained from the same slices ($R=0.94$; $p<0.0001$ for the α4 subunit (Fig. 1C); and $R=0.59$; $p<0.04$ for the δ subunit (not shown); $n=13$ sample pairs from either PF or VLPO region; Spearman rank correlation).

When quantified on PD29–30, 19 days after the last AE, mRNA levels for the α4, β3, $\gamma 2_{L/S}$ and δ subunits of $GABA_AR$ were significantly higher in the PF region in the AE than the S group. In contrast, in the VLPO region, only the δ subunit mRNA was higher in the AE than the S group (Table 1).

Immunoreactivity for both α4 and δ subunits also was significantly increased in the PF region in the AE compared to the S group on PD29–30, whereas the levels of these subunits were not significantly different between the two groups in the VLPO region (Fig. 2).

On PD27–28, 17 days after the last AE, the number of spontaneous movements was significantly lower in the last 30-min interval in rats subjected to AE when compared to the S group ($p<0.02$; Fig. 3A). During the first 60 min of recording, rats from both groups had high levels of motor activity, probably due to novelty of the environment, but the number of movements also tended to be lower in the AE than S group.

On PD47, 37 days after the last AE, the number of spontaneous movements also tended to be lower in the AE group (Fig. 3B). Then, on PD51–52, administration of gaboxadol caused a significantly larger decline of the number of movements in the AE than the S group (Fig. 3C). In rats from both groups, gaboxadol caused a transient total loss of motor activity, consistent with its sedative and sleep-promoting properties described previously [20]. The latency to the first period of immobility lasting over 3 min was significantly shorter in the AE than the S rats ($p<0.005$; Fig. 3D).

Interruption of the normal time course of GABAergic system development may disrupt trophic influences of GABA and maturation of neuronal circuits (e.g., [23]). Data also indicate that prenatal AE can cause an increase in the number of cortical $GABA_ARs$ lasting through adulthood [4]. Similarly, we found that both mRNA and protein levels for the α4 and δ subunits and mRNA levels for two additional subunits were elevated in young adult rats subjected to perinatal AE. Importantly, the changes were region-specific in that they occurred in the PF region but were less evident in the VLPO region. A proportional relationship between mRNA levels and immunoreactivity for the α4 and δ subunits suggested that both transcriptional and translational mechanisms were involved in upregulation of these subunits in rats subjected to perinatal AE. Since the posterior hypothalamus also regulates food intake, it seems possible that regional molecular changes can be caused by reduced caloric intake during the AE. It remains uncertain though whether GABAergic system can be affected. Data from adult rats indicate that caloric deprivation does not alter hypothalamic mRNA levels for GABAergic precursor, glutamic acid decarboxylase, whereas neuropeptide Y mRNA is elevated [28].

A number of previous studies reported an increase in the brain α4 subunit expression following acute or chronic AE, both *in vivo* and *in vitro* (reviewed in [42]). Data show that the α4-β3-δ subunit combination can create a $GABA_AR$ that is extremely sensitive to alcohol at low concentrations [34,40], although in a recent study, the uniquely high sensitivity of such recombinant receptors to alcohol has been questioned [6]. The $GABA_ARs$ containing these subunits are often extrasynaptic and mediate tonic inhibition [5,26,30,40]. Recent data indicate that extrasynaptyc $GABA_ARs$ containing the putative alcohol-sensitive combinations, α4 (or α6) and δ subunits, mediate effects of gaboxadol, a sleep-promoting $GABA_A$ agonist [5,26, 41], and that β3 subunit is also important for the regulation of sleep [37]. Of these subunits, the α6 subunit was not investigated in our study because it is very weakly expressed in the hypothalamus [24].

Thus, perinatal AE results in long-lasting molecular changes in the arousal- and motor activity promoting PF region that can enhance GABAergic inhibition exerted in this region. Our functional data indicating that motor activity is reduced in AE rats may be explained by overexpression of posterior hypothalamic $GABA_ARs$. Since upregulation of $GABA_ARs$ occurs

in the PF region when sleep drive is increased [37], reduced motor activity in the AE rats suggests that their sleep and sleepiness are also increased. Our results demonstrate that AE rats have reduced spontaneous motor activity 17 days after AE and exhibit the same trend 37 days after the insult. In addition, gaboxadol administered 41 days after AE suppressed motor activity more powerfully in the AE group, and AE rats developed immobility more rapidly than the rats of the S group. This is consistent with the possibility of increased inhibition mediated by α4 and δ subunit containing $GABA_A$Rs because we found that both mRNA and protein levels for these subunits were elevated in the arousal- and motor activity-promoting PF region in the AE rats, and gaboxadol can act preferentially through $GABA_A$Rs that include these subunits [5,26,41]. These changes can underlie an impaired regulation of motor activity and sleep-wake behavior that last long beyond the period of the perinatal exposure to alcohol.

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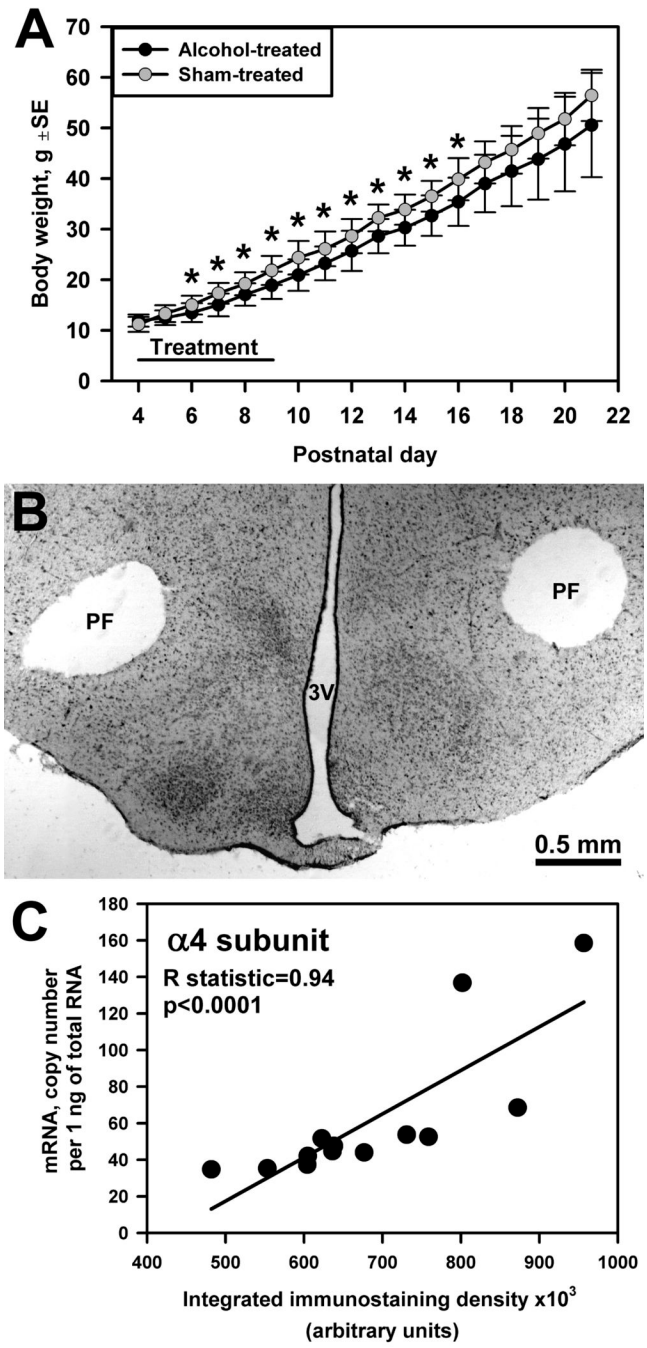

Fig. 1.

(A) Group mean body weight in alcohol- and sham-treated rats (n=11–23 per group) from the beginning of treatment on PD4 to weaning (*, $p<0.05$ between groups). (B) Neutral red-stained section from a posterior hypothalamic slice showing the location of 700 μm tissue micropunches taken bilaterally from the PF region (PF) (3V, third ventricle). (C) Positive correlation between integrated density of α4 subunit dot-blot immunostaining in hypothalamic tissue samples extracted from one side of the slices containing the PF or VLPO region and α4 subunit mRNA levels in samples taken from the opposite side of each slice (data from 13 pairs of samples from 7 sham-treated rats).

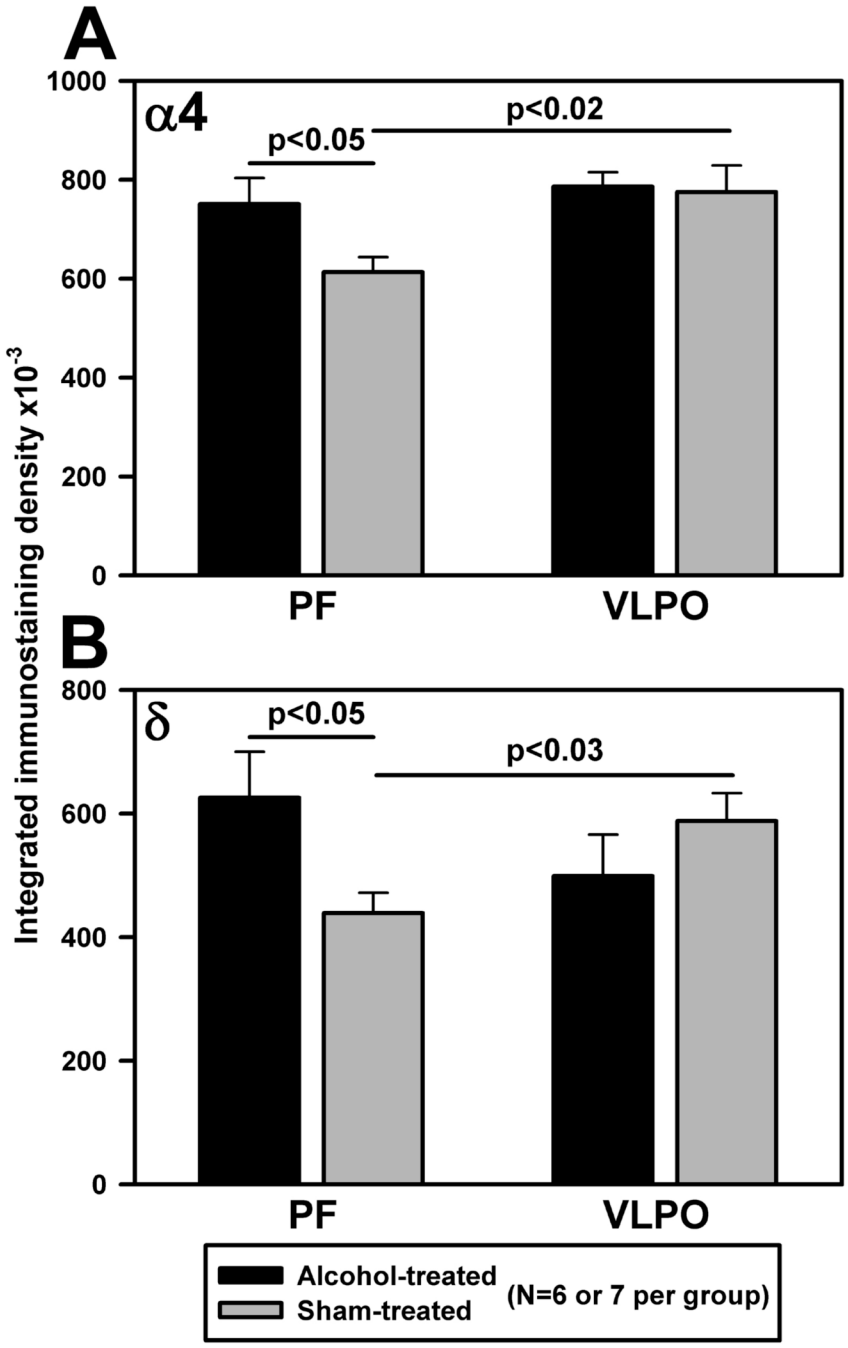

Fig. 2.

Neonatal exposure to alcohol leads to increased immunoreactivity for α4 (A) and δ (B) subunits of $GABA_A$ receptor in the PF region in 29–30 day-old rats.

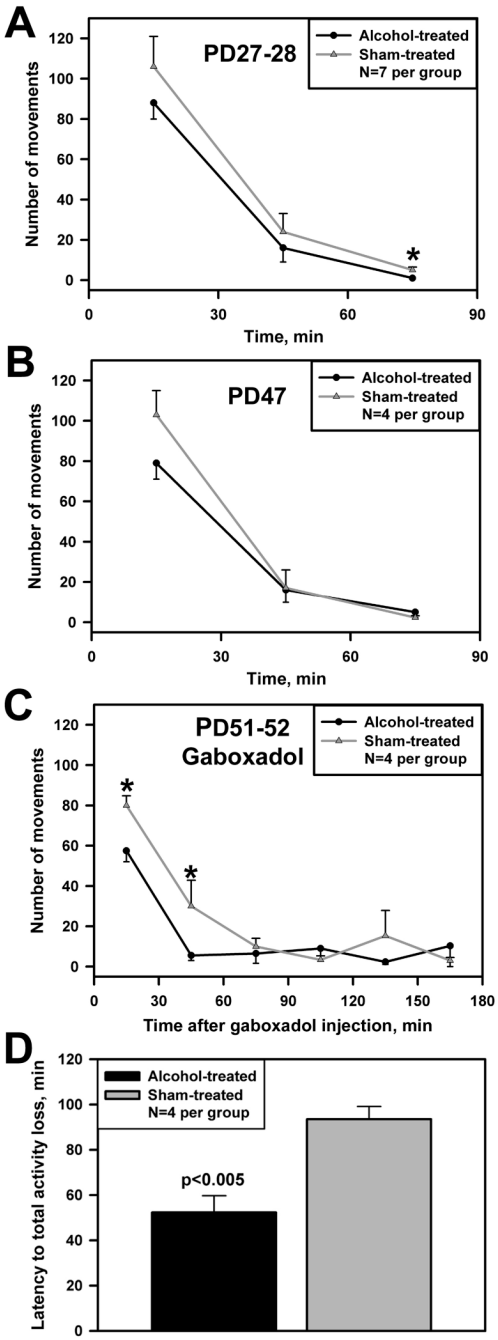

Fig. 3.

Neonatal exposure to alcohol results in reduced spontaneous motor activity in 27–28 day-old rats (A), with the same trend also present on PD47 (B) (*, $p<0.05$ between groups). Following gaboxadol treatment (5 mg/kg, s.c.) on PD51–52, motor activity is reduced more profoundly (C), and the latency to gaboxadol-induced transient period of immobility lasting at least 3 min is shorter in the alcohol- than sham-treated rats (D).

Table 1

Mean $GABA_A$ receptor subunit mRNA levels[a] measured in the perifornical (PF) and ventrolateral preoptic (VLPO) regions in alcohol- and sham-treated rats on postnatal day 29–30.

$GABA_AR$ subunit mRNA	Alcohol-treated		Sham-treated	
	PF	VLPO	PF	VLPO
	(n=6)	(n=6 or 7)	(n=7)	(n=6 or 7)
α1	16,000±1,100	18,400±1,300	14,000±1,100	18,300±2,200
α2	8,500±400	7,700±600	8,100±800	7,100±1,200
α3	10,700±600	10,500±1,100	10,400±700	9,400±500
α4	61±6 *	76±10	42±3	88±17
α5	5,000±600	2,300±50	4,800±600	2,300±190
β1	10,000±1,100	6,500±200	9,700±1,100	7,200±900
β2	24,000±3,700	26,000±1,500	19,000±1,700	26,000±2,900
β3	84,000±12,200 *	66,000±4,000	58,000±2,200	66,000±7,000
γ2	580±37 *	1,800±280	450±20	1,800±500
δ	2.46±0.34 *	49±11 *	1.6±0.2	21±4
ε	150±14	27±5	150±30	36±4

[a] Expressed as cDNA copy numbers per 1 ng of total RNA in tissue sample ± SE.

* $p<0.05$ for differences between alcohol- and sham-treated rats.