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Perinatal exposure to alcohol disturbs spatial learning and glutamate transmission-related gene expression in the adult hippocampus

Mathias Zink, Thomas Ferbert, Stefanie T. Frank, Patricia Seufert, Peter J. Gebicke-Haerter and Rainer Spanagel
Central Institute of Mental Health, Department of Psychiatry and Psychotherapy and Department of Psychopharmacology, University of Heidelberg, PO Box 12 21 20, D-68072 Mannheim, Germany

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Abstract

Perinatal exposure to alcohol (PEA) induces general developmental and specific neuropsychiatric disturbances accompanied by disturbed synaptic plasticity. Here we studied the long-term behavioral consequences of PEA and investigated glutamate transmission-related genes in a longitudinal fashion. After delivery, female Wistar rats and their pups were exposed to ethanol until postnatal day (PD)8 in vapor chambers. At the age of 5 months, the animals were behaviorally characterized. At both PD8 and after the behavioral testing we examined the expression of the vesicular glutamate transporter 1 and excitatory amino acid transporter (EAAT)1–4, as well as the *N*-methyl-D-aspartate receptor subunits (NR)1 and 2A–D, and in parallel receptor binding using ^{3}H-dizocilpine maleate receptor autoradiography. We found highly significant reductions of body weight and length following PEA in pups at PD8. These alterations disappeared in adulthood, when no changes of motor activity and only subtle differences of anxiety-related behavior were observed. It also did not affect T-maze learning, but had a pronounced effect on hippocampus-dependent spatial learning (Morris water maze testing). This specific learning deficit was accompanied by a dysregulation in hippocampal gene expression (significant induction of vesicular glutamate transporter 1, EAAT1, EAAT3, NR2A, 2B, 2C and 2D). Most of the examined genes turned out to be dysregulated to a higher degree at the age of 5 months. We therefore conclude that perinatal ethanol toxicity alters the plasticity of neurodevelopment and the regulation of glutamatergic gene expression, which may result in specific hippocampus-dependent learning deficits in adulthood.

Introduction

Perinatal exposure to alcohol (PEA) induces general developmental and specific neuropsychiatric deficits (Olney *et al.*, 2002; Burd *et al.*, 2004; Bailey & Sokol, 2008). The classical triad of the fetal alcohol syndrome (Lemoine *et al.*, 1968; Jones & Smith, 1973) comprises growth retardation, craniofacial malformations and neurocognitive deficits (Sulik, 2005). Several subsyndromes are determined by the time window and amount of exposure, and characterized according to a four-digit code (Astley & Clarren, 2001; Chasnoff *et al.*, 2010) that quantifies the four key diagnostic features, i.e. growth deficiency, fetal alcohol syndrome facial phenotype, brain dysfunction and gestational alcohol exposure. The alcohol-related neurodevelopmental disorder (Guerri *et al.*, 2009; Kodituwakku, 2009) is attributed to exposure during the last trimester of human pregnancy, corresponding to the first postnatal week of rat development (Dobbing & Sands, 1979).

The pathogenesis of alcoholism in general (Spanagel, 2009) and PEA in particular involves multiple neurobiological systems. The developing hippocampal formation is specifically sensitive to ethanol (Mameli *et al.*, 2005; Klintsova *et al.*, 2007; Miki *et al.*, 2008) and a high importance has been attributed to altered glutamate signaling (Olney *et al.*, 2001; Moriguchi *et al.*, 2007; Vengeliene *et al.*, 2008).

Glutamate, the major excitatory neurotransmitter in the adult mammalian brain, is transported into synaptic vesicles by vesicular glutamate transporters (vGluT1–3) (Fremeau *et al.*, 2004; Shigeri *et al.*, 2004; Nemeroff & Vale, 2005) and upon release interacts with ionotropic or metabotropic receptors (Corlew *et al.*, 2008). The ionotropic *N*-methyl-D-aspartate receptors (NMDARs) form heterotetrameric complexes consisting of two obligatory NR1 subunits and two NR2 subunits (2A, 2B, 2C and 2D), respectively (Paoletti & Neyton, 2007). The specific subunit composition influences the pharmacological and biophysical properties of the receptors (Gielen *et al.*, 2009). Excitatory amino acid transporters (EAAT1–5) control glutamate concentrations in the synaptic cleft by transmembrane reuptake into neurons and glial cells (Danbolt, 2001). EAAT1 (rodent nomenclature – glutamate aspartate transporter) and the most abundant transporter EAAT2 (rodent nomenclature – glutamate transporter 1) are mainly expressed on astroglial cells in the hippocampus, cortex and basal ganglia (Torp *et al.*, 1994; Gadea & Lopez-Colome, 2001) and at lower levels in neurons (Holmseth *et al.*, 2009), in particular during development (Sutherland *et al.*, 1996; Furuta *et al.*, 1997), and in excitatory axonal terminals (Minelli *et al.*, 2001; Chen *et al.*, 2004).

Correspondence: Prof. Dr Mathias Zink, as above.
E-mail: mathias.zink@zi-mannheim.de

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The PEA-induced alterations in the glutamate system might be part of a general modification of synaptic plasticity leading to a premature stabilization of excitatory synapses and strengthening of glutamatergic neurotransmission (Galindo *et al.*, 2005). Acute exposure to ethanol blocks NMDARs (Lovinger *et al.*, 1989; Hundt *et al.*, 1998; Ren *et al.*, 2003) and a hyperglutamatergic state may ensue (Chefer *et al.*, 2011; Spanagel, 2010), which thereby triggers a wave of apoptotic neurodegeneration (Ikonomidou *et al.*, 1999, 2000; Wozniak *et al.*, 2004). Long-lasting effects were determined in the impaired induction of NMDAR-dependent long-term potentiation (Izumi *et al.*, 2005) and reduced sensitivity to *N*-methyl-D-aspartate (Morrisett *et al.*, 1989). Several experimental models of PEA evaluated the glutamatergic effects and, in organotypic cortical slice cultures, we showed induced expression of EAAT1 and EAAT2 (Zink *et al.*, 2004). So far, the results concerning EAAT suggest increased expression of transporters and glutamate uptake (see Supporting Information Table S1) and differential effects on NMDAR subunit (NR) expression as well as binding site availability (see Supporting Information Table S2). Due to differences between species, modalities, doses and time windows of ethanol exposure, as well as variations between brain regions of interest, the findings are to some extent inconsistent. Because PEA-associated molecular effects might contribute to the pathological behavioral phenotypes, they have to be defined in a longitudinal perspective. Therefore, we developed a vapor inhalation model of PEA. We evaluated the gene expression of glutamate transporters and NRs immediately after exposure and in the adult brain. These studies on gene expression were paralleled by a comprehensive behavioral characterization of the rats at 5 months after PEA.

Materials and methods

Animals

Wistar Han outbred rats (Janvier, France) were housed in standard rodent cages (Eurostandard Type IV; Techniplast, Germany) on a 12-h light/dark cycle with lights on at 07:00 h. Animals were provided with food (V1536-R/M-H, extrudate; SSNIFF, Soest, Germany) and water *ad libitum*. All experimental procedures were approved by the respective Committee on Animal Care and Use (reference number of the Regierungspraesidium Karlsruhe: 35-9185.81/G-33/05) and carried out following the local Animal Welfare Acts and in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Ethanol exposure in vapor chambers

Female and male rats were kept under standard conditions for a period of 2 weeks and paired at the age of 14 weeks for 7 days. After separation, eight females were kept singly in air-tight cages of 8.1” height, 9.5” width and 17.0” length within the vapor chamber apparatus (La Jolla Alcohol Research Inc., USA) during the whole period of pregnancy, delivery and the first postnatal week. Water was provided through the lid of the air-sealed cage. For vapor chamber exposure (Zink *et al.*, 2009), ethanol flowed from a large reservoir to a peristaltic pump (FMI Laboratory pump, QC-6; Fluid Metering Inc., Syosset, NY, USA), from which it was carried to a side-arm flask at a flow rate that could be regulated (12 drops/min). This flask was placed on a heater (Barmstead Electrothermal, USA) so that the drops of ethanol hitting the bottom were vaporized. Airflow controlled by a pressure gauge was delivered to the flask and carried the ethanol vapors to the individual boxes with a volume flow of 15 standard cubic feet/h (Dwyer Instruments, MI, USA). Ethanol application had been titrated in pilot studies regarding the air flow and duration of exposure. Marked differences had been observed in the duration of exposure between males, females and pregnant females (Zink *et al.*, 2009). It is of note that the metabolic capacities of rodents differentiate during postnatal development and alcohol dehydrogenase activity markedly increases (Bhalla *et al.*, 2005). In this series, we confined the period of exposure (3.0 h/day) to the first postnatal week, thereby mimicking alcohol exposure during the late phase of neurodevelopment in the last trimester of human pregnancy (Dobbing & Sands, 1979), and obtained average serum ethanol levels in the pups at day 8 immediately after exposure of 176.4 ± 96 mg/dL. Control animals were placed in identical boxes, but only exposed to normal air (delivered at the same rate – 15 standard cubic feet/h). At the end of vapor chamber exposure [postnatal day (PD)8], a subgroup of pups ($N = 8$ in both groups) were killed by decapitation for molecular studies and the littermates were returned to conventional home cages for neurobehavioral analysis starting at the age of 5 months.

Measurement of blood alcohol levels

Blood alcohol levels were measured in 5 μL serum samples using an enzyme-based photometric assay (Ethanol FS, DiaSys; Rolf Greiner BioChemica GmbH, Flacht, Germany). We used venous blood samples of pups killed by decapitation. The reaction is based on the oxidation of ethanol with nicotinamide adenine dinucleotide to acetaldehyde catalyzed by the enzyme alcohol dehydrogenase. Under the conditions of the assay, the extinction of nicotinamide adenine dinucleotide at a wavelength of 376 nm is directly proportional to the amount of ethanol in the sample. Multiple point calibration was performed for each set of samples with reagents provided by DiaSys. By a linear algorithm, the ethanol concentration in the sample of interest can be determined.

Behavioral assessments

The behavioral analysis was performed with 5-month-old male animals of the same litters as the animals used for *in situ* hybridization (PEA: $N = 9$; controls: $N = 13$). Rats had grown up in standard group cages (size IV), had been regularly handled and had received food and water *ad libitum*. Performance in a battery of neurobehavioral tests assessing locomotor activity, anxiety, exploratory behavior and learning was evaluated by a video-camera fixed in a central position at the ceiling of the room and the ETHOVISION software version 3.1 (NOLDUS).

Open-field test

Individual animals were placed into a black rectangular area with side length of 90 cm at a defined edge. At an illumination of 40 lx (temperature 23 °C, humidity 50%), animal behavior was monitored for a total of 10 min. Parameters of analysis were entries to the internal zones (70 × 70 and 50 × 50 cm), duration in these zones, velocity and total distance moved.

Elevated plus maze

The maze was constructed of black Plexiglas, consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 × 40 cm), and was elevated 50 cm off the floor by four steel legs, quite similar to previously described devices (Gonzalez & File, 1997; Osborn *et al.*,

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1998). At an illumination of 184 lx (temperature 22.5 °C, humidity 50%), the animals were placed on the central square with the head looking into one closed arm. Behavior was monitored for a total of 5 min. Parameters of analysis were first occurrence and number of entries into the open and closed arms, further duration, velocity and total distance moved.

T-maze

We adopted the method described by Nagahara & Handa (1999) and constructed a T-maze apparatus of black Plexiglas consisting of a start arm (200 cm) and two goal arms (50 cm). After initial habituation to the apparatus with food pellets (45 mg) distributed in the goal arms, rats were trained in the alternation task and learned to obtain reward from defined places at the end of the goal arms (10 runs/animal/day, training over a period of 6 days, rewards shifting between the goal arms). We then introduced a 45 s delay between the runs and evaluated, over the complete series of testing without and with delay, the duration of trials, direction of shifting at the end of the start arm, mode of shift–shift or shift–stay behavior and number of rewards obtained.

Morris water maze

In the Morris water maze paradigm (Morris & Morris, 1984; D'Hooge & De Deyn, 2001), the animals had to learn to find an invisible, hidden platform with a surface area of 10 × 10 cm that was submerged (−2.5 cm) in water (23 °C) in a circular, black basin (diameter 1.5 m), under illumination of 120 lx, at room temperature (23 °C) and 50% humidity. High-contrast cues were fixed on the walls of the test room. After swimming, the rats rested for 15 s on the platform before they were toweled off and placed back into their home cages. Four trials with changing start positions were performed on each of the first 3 days, each separated by a 60 min inter-trial delay (home cage). On day 4, we performed a probe trial starting from opposite the meanwhile removed hidden platform. Escape latencies were defined as the time between the start of swimming and climbing the platform. We further assessed time periods spent in the circle segments, velocity and total distance moved.

Semi-quantitative analysis of gene expression

At PD8, subgroups of the offspring were killed immediately after finishing the ethanol exposure session. Littermates were killed at the age of 6 months after finalization of behavioral assessments by decapitation. Brains were shock-frozen in isopentane before being stored at −80 °C. According to stereotaxic coordinates of the developing (Paxinos *et al.*, 1994) and adult (Paxinos & Watson, 1986) rat brain, coronal sections (20 μm) were cut in a cryostat at the levels of the anterior cingulate cortex (PD8 – plate 151; adult – plate 12) and dorsal hippocampus (PD8 – plate 168; adult – plate 33). The sections were thaw-mounted on superfrost plus microscopic slides, fixed in 4% paraformaldehyde, dehydrated in ethanol and stored at −20 °C. *In situ* hybridizations (Zink *et al.*, 2005, 2007) were performed on two sections of each animal ($n = 8$/cohort) with ^{35}S-UTP-labeled cRNA probes (see Table 1). Subcloned cDNAs were *in vitro* transcribed using Sp6 or T7-RNA polymerases (MBI-Fermentas, St Leon Roth, Germany). The efficiency of ^{35}S-UTP incorporation was determined and hybridizations with antisense and sense probes at concentrations of 10^7 cpm/mL were carried out at high-stringency conditions (50% formamide, 55 °C) for 16 h. After several washing

TABLE 1. Genes of interest

Gene	GenBank accession number	Sequence	Size of ISH probe (NT)
vGluT1	U_07609	296–720	425
EAAT1	S_59158	2834–3229	415
EAAT2	X_67857	1190–1626	436
EAAT3	D_63772	2092–2692	600
EAAT4	U_89608	1417–1821	405
NR1	U_08266.1	2472–2996	525
NR2A	M_91561.1	4035–4585	551
NR2B	M_91562	4030–4444	415
NR2C	D_13212.1	1878–2419	542
NR2D	NM_022797.1	2896–3413	518

The table summarizes the genes analyzed in this project by semi-quantitative radioactive *in situ* hybridization (ISH) and provides information about the subcloned cDNA fragments used for *in vitro* transcription and their position in published sequences (GenBank accession numbers). The EAAT2 probe is located in a conserved region in order to detect both transcript variants of this gene (Autry *et al.*, 2006). NT, nucleotides.

steps including RNAse A digestion, slices were dehydrated and exposed to X-ray films (Biomax MR1, 18 × 24 cm). After several washing steps including RNAse A digestion, slices were dehydrated and exposed to X-ray films (Biomax MR1, 18 × 24 cm) for the specific necessary periods of time (vGluT1, EAAT1 and EAAT2: 1 day; EAAT3: 5 days; EAAT4 and NR1: 3 days; NR2A: 3 days; NR2B: 3 days; NR2C: 4 days; NR2D: 6 days).

N-methyl-D-aspartate receptor autoradiography

In vitro receptor autoradiography of *N*-methyl-D-aspartate-binding sites was performed according to the method of Zilles *et al.*(1991, 1999), as described elsewhere (Schmitt *et al.*, 2003; Segnitz *et al.*, 2011). The preincubation of the glass-mounted cryostat sections (20 μm, no fixation) for the removal of endogenous ligands was carried out with 50 mM Tris–HCl buffer (pH 7.5) for a total of 15 min (three times for 5 min) at room temperature. Binding assays were performed in the same magnesium- and zinc-free buffer in the presence of 30 mM glycine and 50 mM spermidine with 5 nM [3H]-dizocilpine maleate (Amersham) at 22 °C for 60 min. Two wash steps in incubation buffer, followed by one in *aqua bidestillatum* followed at a temperature of 4 °C. In adjacent sections, non-specific binding was determined by adding 100 μM dizocilpine maleate (20 000-fold excess) in the incubation solution. Typically, non-specific binding of <5% of the total binding was found under these conditions. Tritium-labeled sections were exposed on [3H]-sensitive films (Kodak MS with amplifier screens) for 4 weeks. For quantification, non-specific binding was subtracted from total binding to obtain specific binding. The gray value images of the co-exposed plastic standards were used to compute a calibration curve by non-linear, least-squares fitting, which defined the relationship between the gray values and concentration of radioactivity. The plastic standards had been calibrated to tissue standards prepared from homogenized brain tissue with known protein content to express binding site densities as fmol/mg protein.

For densitometry, the autoradiographic films were analyzed with a Sony video camera (XC ST 70) and AIS software (Applied Information Systems, Chapel Hill, USA). Regions of interest were the anterior cingulate cortex (comprising Cg1 and Cg2), amygdala, prefrontal cortex (PFC), fronto-parietal cortex, caudate nucleus and putamen, the hippocampal subregions granular layer of the dentate gyrus (DG), pyramidal layers of *cornu ammonis* subregions (CA1 and CA3),

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lateral septal nuclei, medial habenular nuclei (mHB), nucleus accumbens, occipital cortex, retrosplenial granular cortex, parietal cortex (PC), temporal cortex (TC), as well as thalamus (TH). Rat PFC was identified according to experimental results using retrograde projection labeling (Uylings & Van Eden, 1990; Van Eden *et al.*, 1990, 1994, 1998). Gray value images of the co-exposed ^{14}C-plastic standards (Amersham Perkin Elmer, Wellesley, USA) were used to compute a calibration curve by non-linear, least squares fitting, which defined the relationship between the gray values and concentration of radioactivity. Non-specific signals were assessed separately for each section in the white matter separating the hippocampal CA1 and cerebral cortex. These readings were subtracted from gray values in the regions of interest (total binding) resulting in a semi-quantitative determination of mRNA abundance.

For statistical evaluation (SPSS version 18.0), we applied descriptive methods in order to determine the means, medians, SDs and SEMs from the individual semi-quantitative assessment of gene expression, receptor binding or behavioral assessments. We tested for normal distribution using histograms and the Kolmogorov–Smirnov *Z*-test and were able to apply parametric methods. According to a recent review on statistical issues regarding developmental neurotoxicity (Holson *et al.*, 2008), we additionally used the litters as the statistical unit for the comparison of body weight and length. Levels of gene expression or receptor binding were compared on an individual base by two-way ANOVA, whereas results at the ages of PD8 and 5 months were analyzed separately. We calculated the main effects of the variables group and brain region, as well as the interaction between cohort and brain region. Within the ANOVA, the posthoc correction least significant difference was applied. *F*-values, degrees of freedom and levels of significance are reported in the Results. Within the PEA group, parametric correlations between blood alcohol levels at PD8 and gene expression were calculated. The group-specific performance in behavioral tests was compared in two-sided Student's *t*-tests, and changes of escape latencies over time were compared by repeated-measure ANOVA with Greenhouse–Geisser correction. Levels of significance are indicated with asterisks: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Results

Behavioral consequences of perinatal exposure to alcohol

The PEA in rats during the first postnatal week was induced by vapor chamber exposure as a model of alcohol neurotoxicity in the last trimester of human pregnancy (Dobbing & Sands, 1979). After daily exposure for 3 h, we achieved average serum ethanol levels in the pups at day 8 of 176.4 ± 96 mg/dL (minimum 74, maximum 342 mg/dL). At this time point, the pups presented a pronounced and highly significant reduction of body weight [PEA ($n = 46$): 14.2 ± 2.2 g; control ($n = 31$): 17.5 ± 1.7 g, $P \leq 3.78E\text{-}11$] and length (PEA: 6.8 ± 0.4 cm; control: 7.2 ± 0.3 cm, $P \leq 5.19E\text{-}07$). These differences were also significant when we used the litters as statistical units for the comparison and had disappeared at adulthood (PEA: 323.7 ± 20.4 g; control: 326 ± 22.8 g) (Supporting Information Fig. S1). Although the duration of gestation (PEA: 24.2 days; control: 23 days) and litter sizes (PEA: 11.75; control: 11) did not differ between groups, the number of pups in the PEA group was reduced by one animal during exposure. After exposure at day 8, a subgroup of pups was killed for molecular studies and littermates grew up in standard cages with water and food *ad libitum* in order to undergo a battery of neurobehavioral tests assessing locomotor activity, anxiety, exploratory behavior and learning at the age of 5 months.

Open field

The experimental groups did not differ in locomotion over a time frame of 10 min. They covered similar distances at equal velocity within the observation period in the whole area and also within the inner zones with side lengths of 50 and 70 cm. Regarding anxiety-related behavior, we observed no differences in the frequency or duration of entries between PEA animals and controls (Fig. 1A–C).

Elevated plus maze

During the observation period of 5 min, parameters assessed in open and closed arms were compared between groups as a hint for exploratory and anxiety-related behaviors. Locomotion and the frequency of entries into and the duration in open or closed arms did not differ; however, the latency of the first entry into the open compartment was longer in PEA animals (two-sided *t*-test: $P = 0.008$) (Fig. 1D–F).

T-maze

During 6 days of training the durations of trials decreased in both groups. On days 7–9, delays of 45 s were introduced between the trials. We did not observe between-group differences regarding the duration, success in gaining reward, preferences in turning left or right, or strategies of win–shift vs. win–stay (Fig. 2A and B).

Morris water maze

Aiming at comparison of hippocampus-dependent spatial learning, we applied Morris water maze testing and observed during the learning phase (3 days) in both groups a marked decrease of escape latencies, defined as the time period between the start of swimming and entering the platform. Over the whole period of training and also during the probe trial, the groups did not differ in locomotor abilities, as represented by the total distance moved and the velocity of swimming. In the probe trial after removing the platform, PEA animals showed a randomly circling swimming behavior in the attempt to find the platform, the first entry into the circular platform zone occurred significantly later (two-sided *t*-test: $P = 0.028$) and they spent significantly less time in the platform segment (two-sided *t*-test: $P = 0.0018$), revealing impaired spatial learning (Fig. 2C and D).

Expression of glutamate transporter genes following perinatal exposure to alcohol

Representative patterns of gene expression are provided in the figures and Supporting Information figures. The evaluation of vGluT1 expression at PD8 (Fig. 3A) revealed significant main effects of the interacting variables group and brain region ($F_{1,27} = 11.647$; $P \leq 0.001$). Within the individual brain regions, significant differences were observed in fronto-cortical regions, most prominently in the anterior cingulate cortex (comprising Cg1 and Cg2) ($F = 6.663$; $P \leq 0.022$), fronto-parietal cortex (FPC) ($F = 6.684$; $P \leq 0.020$), fronto-piriforme cortex (FPirC) ($F = 9.681$; $P \leq 0.008$), PFC ($F = 7.099$; $P \leq 0.018$) and lateral septal nuclei ($F = 96.172$; $P \leq 0.001$). Within the PEA group, a significant correlation between blood alcohol levels and vGluT1 gene expression was detected in the DG ($r = 0.0725$, $P = 0.042$). The evaluation of adult vGluT1 expression (Fig. 3B) revealed significant main effects of the interacting variables group and brain region ($F_{1,31} = 22.691$; $P \leq 0.001$). Within the individual brain regions, significant differences were observed in the anterior cingulate cortex (comprising Cg1 and Cg2) ($F = 5.886$; $P \leq 0.027$), amygdala ($F = 10.074$; $P \leq 0.006$), CA1 ($F = 15.111$;

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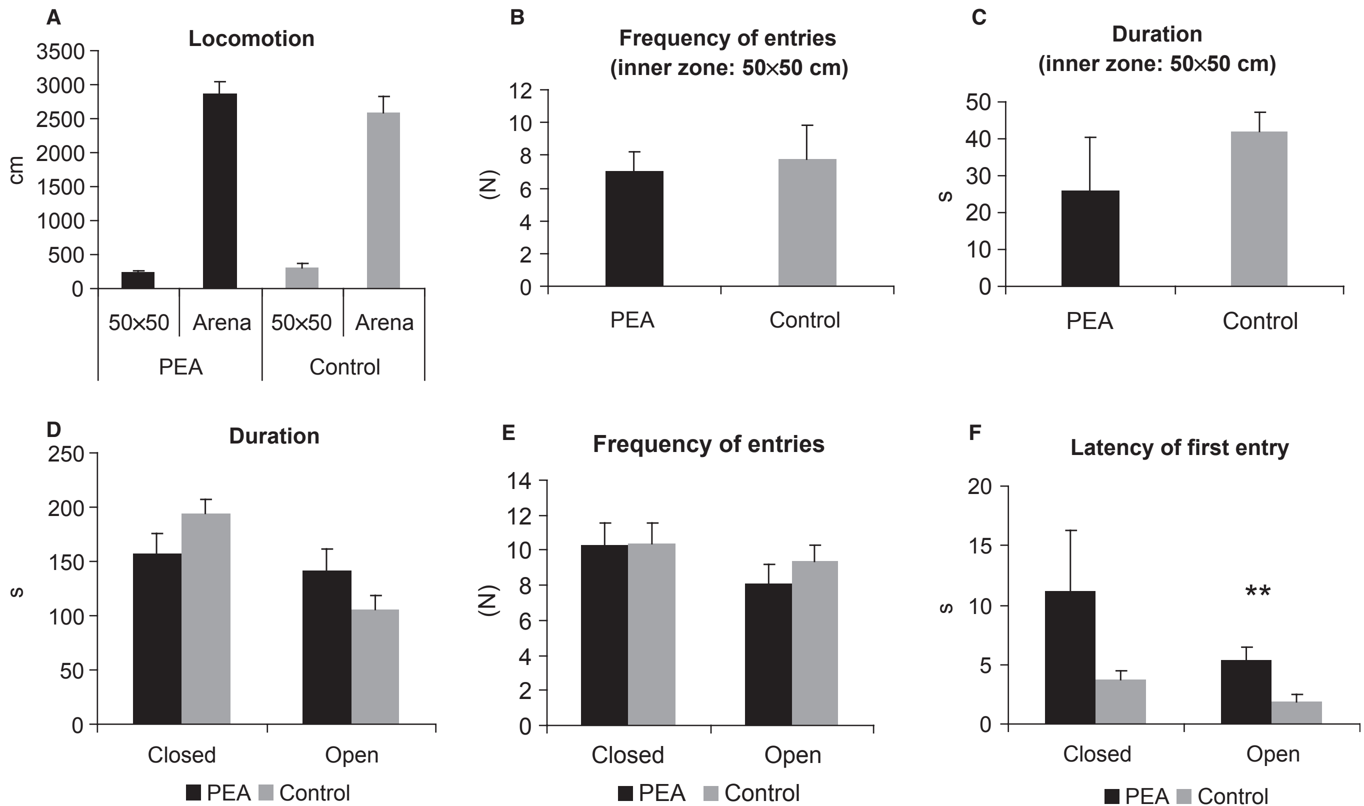

FIG. 1. Behavioral characterization of PEA and control animals was performed at the age of 5 months. In the open-field test, no differences were observed regarding locomotion (A), frequency of entries into the inner zone (B) and time spent in the inner zone (C). Accordingly, the elevated plus maze test showed similar locomotion (D) and frequency of entries to open arms (E) and differences in the latencies to first entry (F). $^{**}P \leq 0.01$.

$P \leq 0.001$), CA3 ($F = 14.185$; $P \leq 0.002$), DG ($F = 7.728$; $P \leq 0.013$), PFC ($F = 6.270$; $P \leq 0.023$), FPC ($F = 7.634$; $P \leq 0.014$), FPirC ($F = 16.375$; $P \leq 0.001$), lateral intermediate septal nucleus ($F = 9.113$; $P \leq 0.009$), mHB ($F = 5.379$; $P \leq 0.034$), occipital cortex ($F = 4.786$; $P \leq 0.044$), PC ($F = 5.171$; $P \leq 0.037$), piriforme cortex (PiC) ($F = 13.308$; $P \leq 0.002$) and TC ($F = 6.939$; $P \leq 0.018$).

The evaluation of EAAT1 expression at PD8 did not reveal significant main effects of the variable group (Fig. 3C). In contrast, adult EAAT1 expression (Fig. 3D) turned out to differ with regard to the interacting variables group and brain region ($F_{1,33} = 6.868$; $P \leq 0.001$). Within the individual brain regions, significant differences were observed in the CA1 ($F = 8.890$; $P \leq 0.009$), CA3 ($F = 5.322$; $P \leq 0.035$), DG ($F = 5.643$; $P \leq 0.030$), PFC ($F = 6.845$; $P \leq 0.019$), mHB ($F = 8.932$; $P \leq 0.009$), occipital cortex ($F = 4.684$; $P \leq 0.046$), PC ($F = 6.039$; $P \leq 0.026$) and retrosplenial granular cortex ($F = 4.680$; $P = 0.046$).

The evaluation of EAAT2, EAAT3 and EAAT4 expression at PD8 did not reveal significant main effects of the variable group. Adult EAAT2 expression revealed significant main effects of the interaction between group and brain region ($F_{1,33} = 2.942$; $P \leq 0.001$). Within the individual brain regions, no significant differences were observed between groups (Supporting Information Fig. S2A). The evaluation of adult EAAT3 (Supporting Information Fig. S2B) expression revealed significant main effects of the interaction between group and brain region ($F_{1,35} = 36.813$; $P \leq 0.001$). Within the individual brain regions, significant differences were observed in the CA3 ($F = 5.266$; $P \leq 0.036$), PiC ($F = 4.532$; $P \leq 0.049$), retrosplenial granular cortex ($F = 9.301$; $P = 0.008$) and TH ($F = 5.793$; $P = 0.029$). Completing the evaluation of transmembrane transporters, adult EAAT4 expression (Supporting Information Fig. S2C) appeared significantly different with regard to the interaction between group and brain region ($F_{1,33} = 24.393$; $P \leq 0.001$). Within the individual brain regions, significant differences were observed in the caudate nucleus and putamen ($F = 6.731$; $P \leq 0.020$), FPirC ($F = 9.145$; $P = 0.008$) and TH ($F = 5.901$; $P = 0.027$).

At PD8, no significant differences in NR expression were observed in the regions of interest (Figs 4A and C, and 5A, D and E). In contrast, adult NR1 expression (Fig. 4B) revealed significant main effects of the interaction between group and brain region ($F_{1,33} = 10.203$; $P \leq 0.001$). Within the individual brain regions, significant differences were observed in the FPirC ($F = 4.777$; $P \leq 0.044$), PiC ($F = 7.926$; $P \leq 0.012$) and TC ($F = 4.890$; $P \leq 0.042$). The evaluation of adult NR2A expression (Fig. 4D) revealed significant main effects of the interaction of the variables group and brain region ($F_{1,31} = 14.989$; $P \leq 0.001$). Within the individual brain regions, significant differences were observed in the amygdala ($F = 5.817$; $P \leq 0.028$), CA1 ($F = 8.293$; $P \leq 0.011$), CA3 ($F = 5.827$; $P \leq 0.028$), FPirC ($F = 6.919$; $P \leq 0.018$), PiC ($F = 6.782$; $P \leq 0.019$), TC ($F = 5.404$; $P \leq 0.034$) and TH ($F = 7.717$; $P \leq 0.013$). The evaluation of adult NR2B expression (Fig. 5B) resulted in significant main effects of the interaction between group and brain region ($F_{1,33} = 21.796$; $P \leq 0.001$). Within the individual brain regions, significant differences were observed in the CA1 ($F = 9.763$; $P \leq 0.007$), CA3 ($F = 6.973$; $P \leq 0.018$), DG ($F = 7.376$; $P \leq 0.015$), mHB ($F = 5.274$; $P \leq 0.035$), PiC ($F = 5.955$; $P \leq 0.027$) and TH ($F = 5.517$; $P = 0.032$). The evaluation of adult NR2C expression (Fig. 5D) revealed significant main

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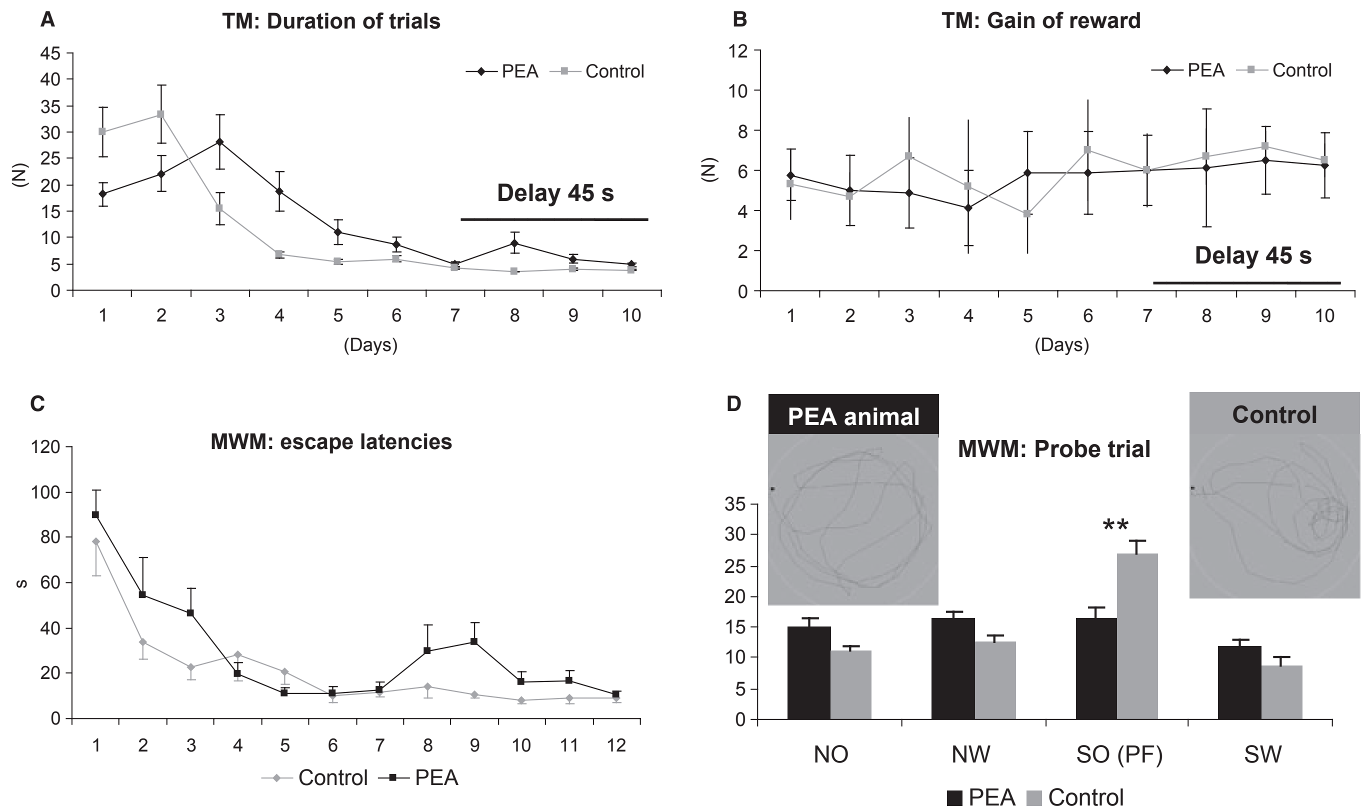

FIG. 2. Two learning paradigms were applied. In the T-maze, PEA animals and controls did not differ regarding latencies during training sessions and after the introduction of a delay between the runs (A) as well as the number of gained rewards (B). In contrast, hippocampus-dependent learning was found to be severely impaired. Although both groups reduced the latencies during training (C), in the probe trial a significantly higher preference for the platform area became apparent in control animals (D). The different strategies in searching the platform are visualized by the representative swimming courses. $^{**}P \leq 0.01$; MWM, Moris water maze; NO, north-eastern sector; NW, north-western sector; PF, platform; SO, south-eastern sector containing the platform (PF); SW, south-western sector; TM, T-maze.

effects of the interaction between group and brain region ($F_{1,29} = 4.034$; $P \leq 0.001$). Within the individual brain regions, significant differences were observed in the PFC ($F = 6.554$; $P \leq 0.021$), fronto-parietal cortex ($F = 4.884$; $P = 0.042$), FPirC ($F = 5.746$; $P = 0.029$), and nucleus accumbens ($F = 6.298$; $P \leq 0.023$). More pronounced effects were detected for adult NR2D expression (Fig. 5F), where the interaction between group and brain region resulted in significant differences ($F_{1,33} = 16.599$; $P \leq 0.001$). Within the individual brain regions, significant differences were observed in the amygdala ($F = 12.535$; $P \leq 0.003$), CA1 ($F = 12.345$; $P \leq 0.003$), CA3 ($F = 9.526$; $P \leq 0.007$), DG ($F = 11.281$; $P \leq 0.004$), mHB ($F = 17.699$; $P \leq 0.001$), occipital cortex ($F = 7.646$; $P \leq 0.014$), PC ($F = 10.627$; $P \leq 0.005$), PiC ($F = 4.898$; $P \leq 0.042$), TC ($F = 10.775$; $P \leq 0.005$) and TH ($F = 8.463$; $P = 0.010$).

N-methyl-D-aspartate receptor binding

We finally evaluated dizocilpine maleate receptor binding and detected at PD8 significant main effects of the interacting variables group and brain region ($F_{1,11} = 8.809$; $P \leq 0.001$) (Fig. 6A). Within the individual brain regions, significant differences were observed in the hippocampus ($F = 6.090$; $P \leq 0.027$), PC ($F = 19.199$; $P = 0.001$) and retrosplenial granular cortex ($F = 5.318$; $P \leq 0.037$). The evaluation of adult dizocilpine maleate receptor binding (Fig. 6B) revealed significant main effects of the interaction between group and brain region (d.f. = 33; $F = 53.486$; $P \leq 0.000$). Within the individual brain regions, significant differences were observed in the PC ($F = 5.068$; $P \leq 0.039$).

Discussion

We report on a PEA model in rats based on alcohol vapor exposure and marked associations of spatial learning deficits with altered hippocampal glutamate neurotransmission-related gene expression. The perinatal period of brain development appears to be highly sensitive to long-lasting effects of alcohol on brain function as we observed progressive modifications of gene expression over time. Hence alterations in gene expression were hardly detectable shortly after PEA but progressed into adulthood with a marked induction of several glutamate neurotransmission-related genes. Although our findings suggest that PEA-induced changes in hippocampal glutamatergic functions underlie disturbed spatial learning in adulthood, it is not possible to directly prove causality without specific pharmacological or genetic rescue experiments.

Perinatal exposure to alcohol in rats produces an alcohol-related neurodevelopmental disorder-like syndrome

Several animal models of PEA successfully applied ethanol via inhalation (Karanian *et al.*, 1986) and assessed the effects on glutamatergic neurotransmission (Bellinger *et al.*, 2002; Sabeti & Gruol, 2008). Ethanol vapor chambers allow a precise regulation of exposure periods and doses (Lee *et al.*, 2000, 2003; Lee & Rivier, 2003), in order to interfere with pre- and postnatal development (Ryabinin *et al.*, 1995).

Our animal model shows robust face and construct validity. Both the developmental retardation and the neurobehavioral phenotype are

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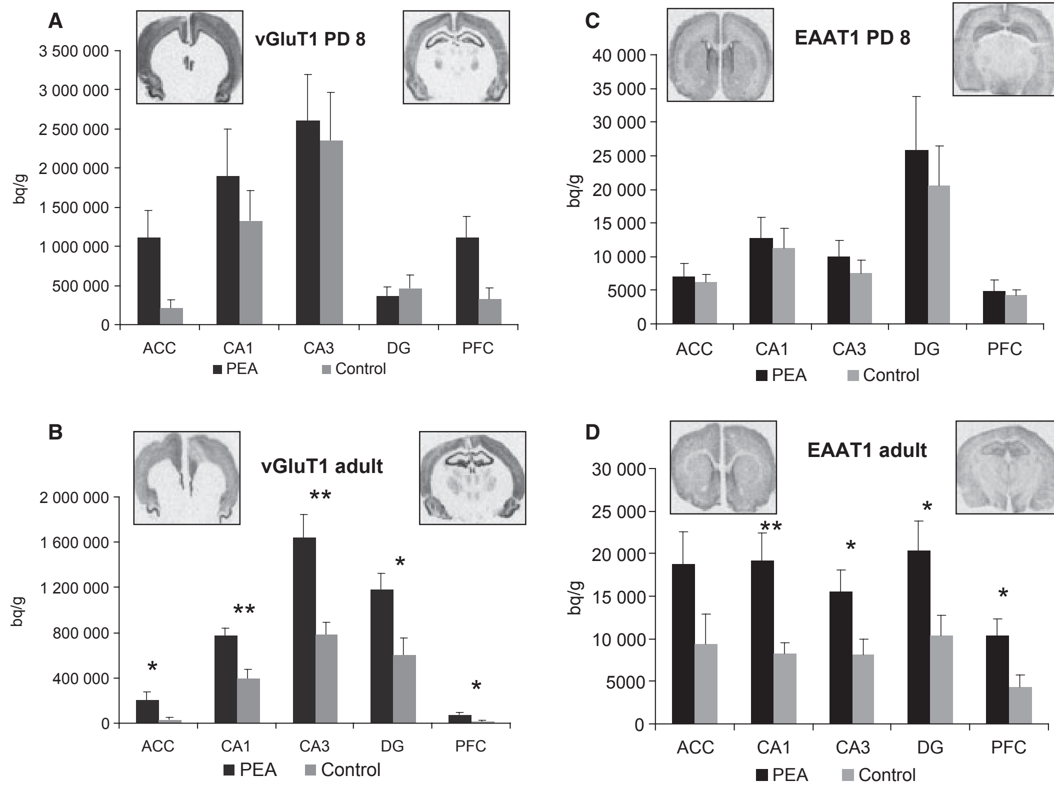

FIG. 3. *In situ* hybridization (ISH) revealed differential expression of vGluT1 (PD8: A; adult: B) and EAAT1 (PD8: C; adult: D) in PEA and controls. Means of the groups are represented by bars ± SEM. Levels of statistical significance are depicted with asterisks: $***P \leq 0.001$, $**P \leq 0.01$, $*P \leq 0.05$. Representative expression patterns at the levels of anterior cingulate cortex (comprising Cg1 and Cg2) (ACC) and hippocampus are shown. bq/g, Becquerel/g.

closely reminiscent of the human phenotype within the fetal alcohol syndrome spectrum including the neurocognitive deficits (Guerri *et al.*, 2009; Kodituwakku, 2009). Within the neurobehavioral characterization, the delay in entering the open arms of the elevated plus maze suggests increased anxiety levels in PEA animals, but corresponding changes were not observed in the open-field testing. Most importantly, we detected a hippocampus-dependent impairment of spatial learning. This effect is in accordance with deficits of ethanol-exposed children and other valid animal models using similar doses and time windows (Bellinger *et al.*, 1999; Mameli *et al.*, 2005; Miki *et al.*, 2008), in particular ethanol-induced Morris water maze deficits observed in rats (Goodlett & Peterson, 1995; Minetti *et al.*, 1996; Goodlett & Johnson, 1997; Kim *et al.*, 1997; Clements *et al.*, 2005), mice (Endres *et al.*, 2005) and guinea pigs (Richardson *et al.*, 2002).

Previous studies were able to define pleiotropic developmental ethanol effects on the glutamatergic system, distinctly depending on the dose and time window of exposure (see Supporting Information Tables S1 and S2). Our gene results continue these concepts and attribute altered expression of glutamate transporters and NRs to the phenotype of disturbed spatial learning.

Glutamate transporter expression

The presynaptic vGluT1 is induced in several hippocampal and cortical regions. The dose–response effect in the DG, represented by a positive correlation of vGluT1 expression to the blood alcohol levels, suggests a causal interaction. Although vGluT1 expression cannot be directly correlated with glutamate release properties it is of note that Iqbal *et al.* (2006) reported on increased glutamate release, whereas other studies did not find changes (Byrnes *et al.*, 2003; Carta *et al.*, 2003) or even a decrease in hippocampal slices (Butters *et al.*, 2000, 2003). However, *in vitro* studies established an induced glutamate uptake by ethanol in astrocyte cultures (Smith & Zsigo, 1996; Smith, 1997; Smith & Navratilova, 1999; Othman *et al.*, 2002) and increased expression of transmembrane glutamate transporters in cortical slice cultures (Zink *et al.*, 2004). Here, we were able to validate those cell and tissue culture results in our animal model and describe a large and significant induction of EAAT1 (and, to a lesser degree, EAAT2 and 3 were also induced in adulthood following PEA) in the adult hippocampus. The upregulation of transmembrane transporters might be a mode of counter-regulation with protective consequences against glutamatergic excitotoxic effects due to an ethanol-induced hyperglutamatergic state (Chefer *et al.*, 2011; Spanagel, 2010) in the developing brain.

N-methyl-D-aspartate receptor subunit expression

We also investigated the NMDAR expression on mRNA and protein levels. The constitutive NR1 subunit appeared nearly unchanged at PD8, but induced in adult brains, whereas the statistical analysis revealed only trends of induction in the hippocampus. More pronounced and significant inductions were observed regarding NR2A, 2B and 2D subunits in the hippocampus (Figs 4 and 5). Because the function of the NMDAR is largely determined by the specific type-subunit composition (Gielen *et al.*, 2009), the described alterations have direct importance for NMDAR function. The increase of the NR2A subunit observed here confirms previous results (Nixon *et al.*, 2004; Toso *et al.*, 2005) and induced hippocampal NR1

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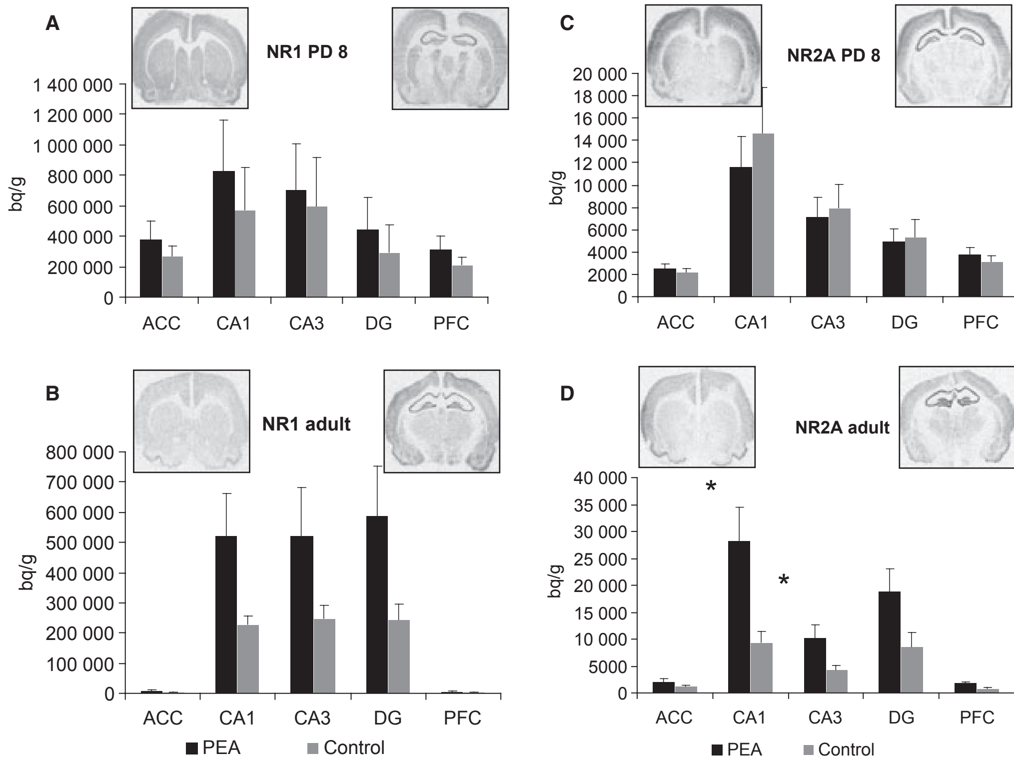

FIG. 4. Differential gene expression of NR1 (PD8: A; adult: B) and NR2A (PD8: C; adult: D) in PEA and controls as assessed by *in situ* hybridization. Means of the groups are represented by bars + SEM. Due to large quantitative differences between hippocampal and cortical expression levels, the measurements of NR1 and NR2A appear downscaled in the anterior cingulate cortex (comprising Cg1 and Cg2) (ACC) and PFC. Levels of statistical significance are depicted with asterisks: $^{**}P \leq 0.01$, $^{*}P \leq 0.05$. Representative expression patterns at the levels of the ACC and hippocampus are shown. bq/g, Becquerel/g.

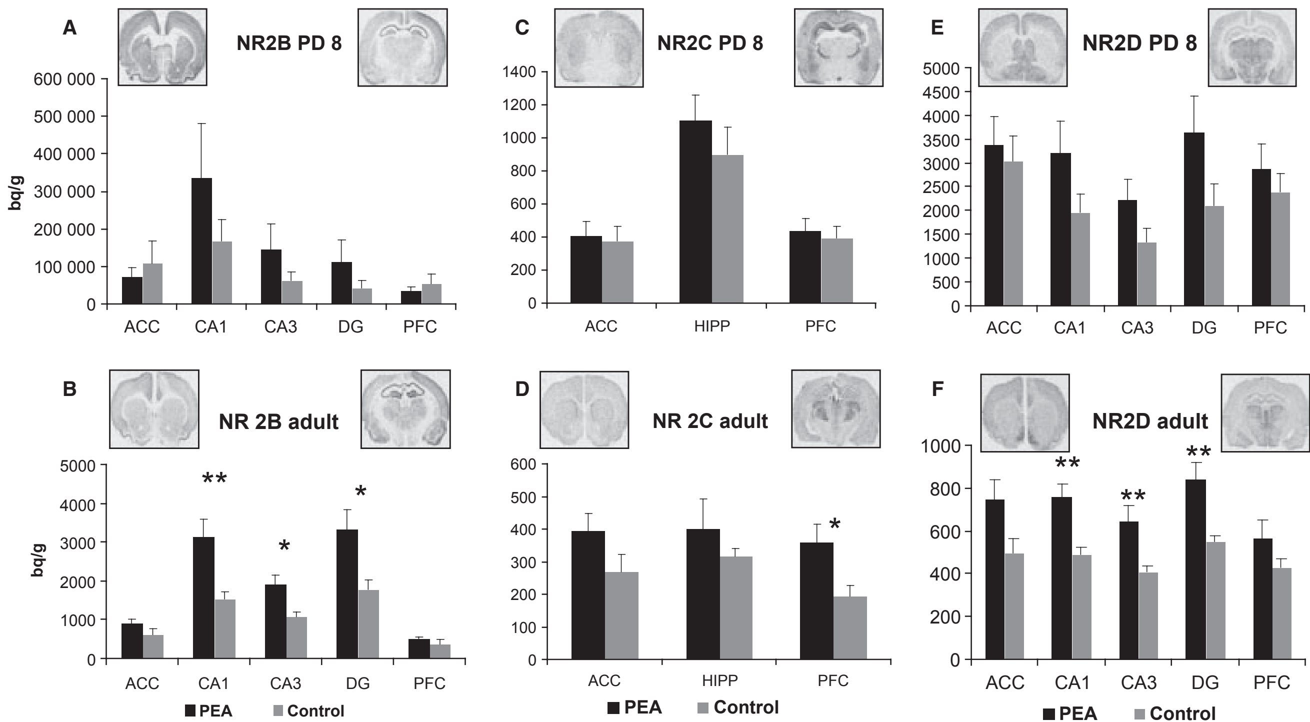

FIG. 5. Differential expression of NR2B (PD8: A; adult: B), NR2C (PD8: C; adult: D) and NR2D (PD8: E; adult: F) in PEA and controls as assessed by *in situ* hybridization. Means of the groups are represented by bars + SEM. Levels of statistical significance are depicted with asterisks: $^{**}P \leq 0.01$, $^{*}P \leq 0.05$. Representative expression patterns at the levels of the anterior cingulate cortex (comprising Cg1 and Cg2) (ACC) and hippocampus (HIPP) are shown. bq/g, Becquerel/g.

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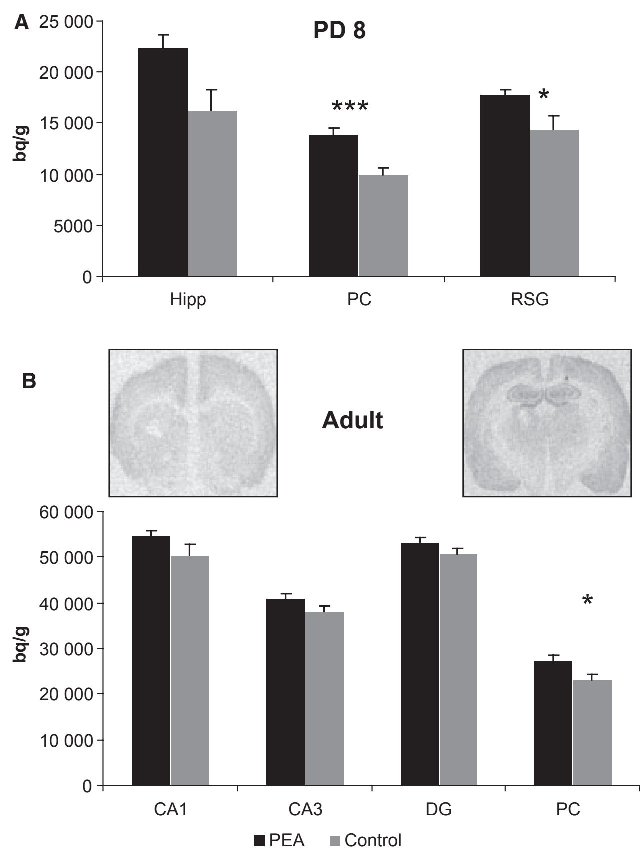

FIG. 6. Effects of developmental exposure to ethanol on NMDAR binding sites at PD8 (A) and in adult rats (B). Means of the groups are represented by bars + SEM. Levels of statistical significance are depicted with asterisks: $***P \leq 0.001$, $*P \leq 0.05$. Typical autoradiograms at the levels of the anterior cingulate cortex (comprising Cg1 and Cg2) and hippocampus of adult rats are shown. bq/g, Becquerel/g. HIPP, hippocampus; RSG, retrosplenial granular cortex.

(Naassila & Daoust, 2002; Iqbal *et al.*, 2006; Samudio-Ruiz *et al.*, 2010) and NR2D (Toso *et al.*, 2006) expression has also been described previously, whereas increased hippocampal NR2B expression has not been reported before. As detailed in Supporting Information Table S2, major differences between model systems render the comparison of PEA effects on NMDAR expression quite difficult. The general pattern of small or lacking acute effects at PD8 in contrast to larger transcriptional effects in the adult brain is in concert with several previous studies where no changes of perinatal NR expression were found (Costa *et al.*, 2000; Bellinger *et al.*, 2002; Byrnes *et al.*, 2003; Nixon *et al.*, 2004). None of these previous studies included a sample of adult animals and only by the use of our longitudinal design were we able to detect this progressive increase in specific gene expression into adulthood. Because neither the constitutive NR1 subunit nor the general availability of NMDAR binding sites appeared induced in adult hippocampus, we assume that toxic effects during early development alter the orchestration of coordinated subunit expression with negative consequences on NMDAR function.

Developmental plasticity

Ethanol exposure during early brain development was found to induce long-lasting and even progressive effects. This observation fits well to the general concept of specific properties of developmental neural plasticity (Zink, 2007). Regarding mechanisms, it seems unlikely that ethanol directly affects the transcription of glutamatergic genes by an interaction with ribosomal proteins or RNA polymerases. According to previously reported findings, it is suggested that initially exposure to ethanol blocks NMDARs (Lovinger *et al.*, 1989; Hundt *et al.*, 1998; Ren *et al.*, 2003), leads to a hyperglutamatergic state (Chefer *et al.*, 2011; Spanagel, 2010), disrupts synaptogenesis in the developing brain (Deng & Elberger, 2003; Olney, 2004), and triggers thereby a massive wave of apoptotic neurodegeneration (Ikonomidou *et al.*, 1999, 2000; Wozniak *et al.*, 2004). It is of note that most of the examined genes turned out to be dysregulated to a higher degree at the age of 5 months, providing a strong argument against the assumption that ethanol toxicity might be confined to acute and transient effects. We therefore propose the hypothesis that ethanol toxicity during critical processes of neurodevelopment alters fundamental set points in the glutamate transmission-related gene cluster. On the molecular level, epigenetic alterations might disturb the orchestration of gene expression with long-lasting consequences, as generally discussed in neuropsychiatric disorders (Ptak & Petronis, 2010), alcoholism (Shukla *et al.*, 2008; Spanagel, 2009) and specifically the fetal alcohol spectrum disorders (Haycock, 2009; Liu *et al.*, 2009; Moonat *et al.*, 2010).

Conclusion

Altered glutamatergic neurotransmission following PEA further supports the notion that ethanol affects and disturbs synaptic functions, in particular in the developing hippocampus (Berman & Hannigan, 2000; Miki *et al.*, 2008). The described molecular alterations add further possible targets to previously described gene alterations for further research and therapeutic interventions. It is a specific strength of our model that it mirrors the extraordinary sensitivity of the perinatal period to toxic influences. Exposure to ethanol might not necessarily alter immediate gene expression, but in our PEA model it changes the closely counterbalanced processes of glutamate transport, receptor subunit composition and reuptake, and may thus result in a decline in hippocampal-dependent learning behavior.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. After exposure to ethanol (PD8), a highly significant retardation of growth was found in reduced body weight (A) and shorter body length (B) in comparison to control animals.

Fig. S2. Adult gene expression (*in situ* hybridization) of EAAT2 (A), EAAT2 (B) and EAAT3 (C) in PEA and controls. Means of the groups are represented by bars + SEM. Levels of statistical significance are depicted with asterisks: $*P \leq 0.05$, $***\ P \leq 0.001$. Representative expression patterns at the levels of the anterior cingulate cortex (comprising Cg1 and Cg2) (ACC) and hippocampus are shown. bq/g, Becquerel/g.

Table S1. Effects of PEA on glutamate release and transporter expression.

Table S2. Effects of PEA on NMDAR expression.

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Abbreviations

CA1 and CA3, pyramidal layers of *cornu ammonis* subregions; DG, hippocampal subregion granular layer of dentate gyrus; EAAT, excitatory amino acid transporter; FPC, fronto-parietal cortex; FPirC, fronto-piriforme cortex; HIPP, hippocampus; mHB, medial habenular nuclei; NMDAR, *N*-methyl-D-aspartate receptor; NR, *N*-methyl-D-aspartate receptor subunit; PC, parietal cortex; PD, postnatal day; PEA, perinatal exposure to ethanol; PFC, prefrontal cortex; PiC, piriforme cortex; RSG, retrosplenial granular cortex; TC, temporal cortex; TH, thalamus; vGluT, vesicular glutamate transporter.

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