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Fetal Exposure to GABA-Acting Antiepileptic Drugs Generates Hippocampal and Cortical Dysplasias

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Summary: *Purpose:* The management of epilepsy during pregnancy entails a number of concerns. While seizures may affect adversely maternal and fetal outcome, antiepileptic drugs (AEDs) may increase the incidence of congenital abnormalities and possibly affect postnatal cognitive development in the offspring. Experimental animal studies can aid in assessing teratogenic features associated with individual AEDs and/or with seizures, and to identify the mechanisms involved. The purpose of this study was to investigate the consequences of prenatal exposure to (a) different AEDs and (b) maternal seizures on brain maturational processes in rats.

Methods: Pregnant rats received from embryonic days 14 to 19 intraperitoneal injections of carbamazepine (20 mg/kg/day), vigabatrin (200 mgkg/day), and valproate (100 mg/kg/day) at doses not widely different from those used clinically. Pups exposed to AEDs in utero were analyzed postnatally. Animals born to "kindled" pregnant animals that had experienced one generalized convulsive seizure per day during the same gestational period were analyzed in parallel.

Results: Prenatal exposure to vigabatrin and valproate, which act on GABA signaling, induced hippocampal and cortical dysplasias, which were likely to result from a neuronal migration defect and neuronal death. By contrast, offspring of rats exposed to carbamazepine (which at the dose used produced low plasma concentrations) or to generalized convulsive seizures showed no clear-cut evidence of dysplasias.

Conclusions: We suggest that AEDs that increase the extracellular concentration of GABA might induce severe neuronal migration disorders. Drugs acting through other molecular targets would also perturb cortical maturation. The potential clinical relevance of these results should be a subject of future research.
Key Words: Development—Epilepsy—Neuronal migration—Seizures.

The treatment of epilepsy during pregnancy entails a number of concerns. While maternal seizures may adversely affect maternal and fetal outcome (La Joie and Moshe, 2004), prenatal exposure to antiepileptic drugs (AEDs) may increase the incidence of congenital abnormalities (Samren et al., 1999; Arpino et al., 2000; Holmes, 2002; Perucca, 2005) and possibly affect postnatal cognitive development (Koch et al., 1999; Adab et al., 2001; Gaily et al., 2004; Vinten et al., 2005; Eriksson et al., 2005; Perucca, 2005; Viinikainen et al., 2006). Clinical studies in this area are often difficult to interpret and compare due to a number of confounders (see [Barrett and Richens, 2003]), including the influence of socioeconomical factors and parental educational level on the outcome of exposed children. Moreover, the analysis of the neurodevelopmental consequences of fetal exposure to AEDs has been restricted to the most severe malformations (i.e., spina bifida, microcephaly), and the potential occurrence of subtle brain maturation abnormalities has been poorly investigated, partly because of difficulties in detecting such defects in the clinical setting. Experimental animal studies can be useful in investigating the effects of AEDs and/or seizures on neurodevelopment, and in identifying the potential mechanisms involved.

In the present study, we compared the morphological consequences of prenatal exposure to AEDs and seizures in rats. We focused our examinations on cortical dysplasias, also referred to as cortical malformations, neuronal migration disorders, or cortical dysgenesis, that are among the most frequent pathological findings in pediatric epilepsies (Barkovich and Raybaud, 2004). Our findings indicate that prenatal-exposure to vigabatrin (VGB) and valproate (VPA), which act on GABA signaling (Grant et al., 1991; Loscher, 2002), induces hippocampal and cortical dysplasias in rats. By contrast, offspring of rats

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exposed to kindled-seizures show no clear-cut evidence of dysplasias. Since GABA is the principal excitatory transmitter in the developing brain and acts as an epigenetic factor to control developmental processes (Represa and Ben Ari, 2005), including cortical and hippocampal neuroblast migration (Behar et al., 2000; Manent et al., 2005), we suggest that AEDs that increase the extracellular concentration of GABA may interfere with neuronal migration in the developing brain. The potential clinical relevance of these findings should be ascertained.

MATERIAL AND METHODS

In vivo experiments: treatments

All experimental procedures were performed in agreement with the European Union and French legislation concerning the care and use of laboratory animals. Pregnant Wistar rats (Janvier, Le Genest Saint Isle, France, eight animals per condition) received from embryonic day 14 (E14) (E0 being the day in which positive vaginal smear was observed) to E19, two intraperitoneal (i.p.) injections per day of one of the following AEDs (all from Sigma, Lyon, France; dissolved in PBS – DMSO 5%): CBZ (20 mg/kg/day), VGB (200 mg/kg/day), and VPA (100 mg/kg/day). These doses were not widely different from those used in humans and were chosen according to the literature concerning animal experimentation (see Table 1), avoiding doses that have been reported to be teratogenic (Menegola et al., 1996) or to cause massive apoptotic neurodegeneration in the developing brain (Bittigau et al., 2002). Control (CTL) pregnant females received the same injections with the vehicle only. The way of administration chosen here differs from that of human patients. Though oral administrations may be possible in animals using gavages, they represent an important source of stress, which may also interfere with pregnancy and lead to fetal brain alterations. Liquid diets were not employed either since it is difficult to efficiently control drugs dosages, as liquid is provided ad libitum to the animals. For these reasons, we chose i.p. injections, as the most convenient way to precisely adjust AEDs dosage.

TABLE 1. AEDs dosing

	[1]	[2]	[3]	[4]	[5]	[6]	Present study
CBZ	1.25–15	10–15	40				20
VGB	12.5–50	25–37.5		500–1,000	50–200		200
VPA	5–30	10–60	100–150		50–400	900	100

Doses (mg/kg/day), as used in human epileptic patients ([1]–[2]), experimental animal studies ([3]–[4]), or causing apoptosis or teratogenic effects in animals ([5]–[6]), as compared to those used in the present study.

[1] Dosing in adult epileptic patients, MedlinePlus, Drugs, and Supplements, http://www.nlm.nih.gov/medlineplus/druginformation.html; [2] Dosing in adult epileptic patients, American Epilepsy Society (AES), Antiepileptic Drug Information, http://www.aesnet.org/Visitors/PatientsPractice/aed/index.cfm; [3] Amygdala- and hippocampal-kindled seizures in adult rats (Otsuki et al., 1998); [4] KA-induced status epilepticus in adult rats (Halonen et al., 1995); [5] Apoptotic effects during the first postnatal week in rats (Bittigau et al., 2002); [6] Teratogenic effects during pregnancy in rats (Menegola et al., 1996).

Pregnant females also received a single i.p. injection with the S-phase marker 5-bromo-2′-deoxyuridine (BrdU, 50 mg/kg, dissolved in PBS—NaOH 0.007N, Sigma) at E15. Animals that have been exposed to AEDs in utero were analyzed at postnatal (P) day 0 and at P30. They were deeply anaesthetized with an injection of chloral hydrate (Sigma), perfused intracardially with the fixative solution (4% paraformaldehyde and 0.5% glutaraldehyde in PBS), and their brains were cut coronally (60-μm thickness) with a vibratome (Leica, Nussloch, Germany).

In vivo experiments: pharmacokinetic studies

Plasma concentrations of CBZ and VPA were evaluated in separate groups of pregnant animals (6 animals par condition) treated in the same way as those used to assess for cerebral malformations. Blood samples were collected by tail-venous puncture at 0.5, 2, and 4 h after the first (E14) and last (E19) injection, respectively (see Fig. 1). The plasma was separated within 1 h and frozen at -20°C until assay.

In vivo experiments: kindling

To test the impact of seizures on brain development the kindling model of epilepsy was chosen, as this is the only model that allows controlling the number, the severity, and the duration of seizures during the period of interest (E15-E20). Four female rats were stereotaxically implanted under general anesthesia (diazepam, Roche; 4 mg/kg i.p.; ketamine, Merial; 1,000 mg/kg i.p.) with a bipolar electrode (insulated twisted stainless steel wire—150-μm diameter) aimed at the basal lateral nucleus of the amygdala (AP: 2.7 mm; ml: 4.5 mm; DV: 9.0 mm, with reference to Paxinos and Watson's atlas of the rat brain [Paxinos et al., 1991]). Three monopolar stainless steel electrodes were screwed on the animal's skull over the parietal and occipital (reference) cortex. All electrodes were soldered to a female microconnector and secured to the skull with acrylic cement. Starting 1 week after surgery, the animals were stimulated for 2 s once a day through the amygdala electrode using a monophasic square wave current of 300 μA (frequency = 50 Hz; pulse width = 1 ms). Both behavior and EEG were monitored for 5 min before and after the stimulation. Within 15 days, all animals displayed a stage-5 secondarily generalized clonic seizure, characterized by forelimb clonic movements associated with rearings and fallings (Racine, 1972). For each animal, five stage five seizures were induced before mating. During pregnancy, the animals were stimulated once a day from E15 to E19-20 using the same parameters. For each stimulation, stage four to five seizures were observed.

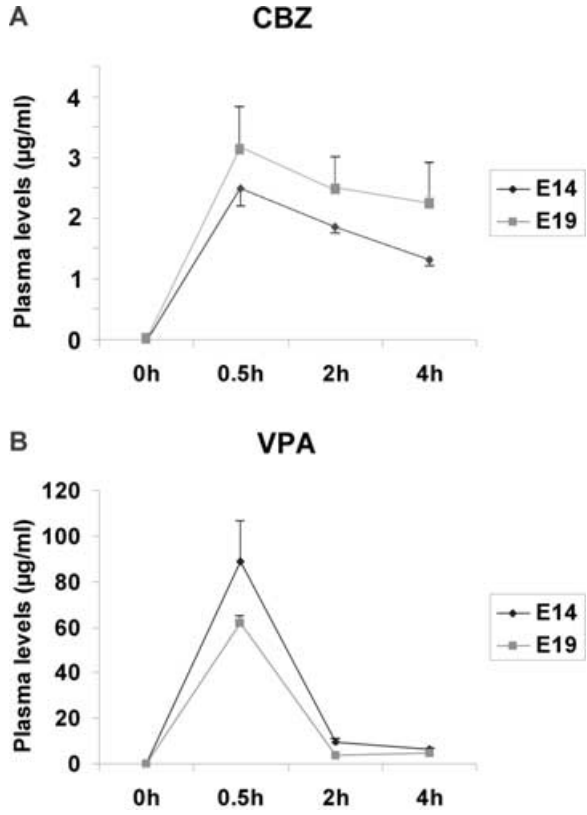

FIG. 1. Plasma concentrations of *CBZ* and *VPA* plasma concentrations, expressed as μg/ml $\pm$ SEM, were determined at 0.5, 2, and 4 h after the morning injection on gestational days E15 and E19. **A.** *CBZ* concentrations were at all times at the lower range of those occurring clinically in patients receiving therapeutic doses (usually quoted optimal range: 4–12 μg/ml [Eadie, 2001]). **B.** Plasma *VPA* concentrations at 30 min were within the range encountered clinically (usually quoted optimal range: 50–100 μg/ml [Eadie, 2001]). When comparing the plasma VPA concentrations found in this study in rats with those encountered during clinical use in humans, it should be considered that reported values reflect the total (unbound plus protein-bound) concentrations. Since unbound fraction of *VPA* in rats (16–18% in young rats and 26–30% in elder rats) (Slattum et al., 1996) is at least twice as high as in humans the concentration of unbound, pharmacologically active VPA in rats is expected to be twice as high as that found in humans at equivalent total drug concentrations.

Drug assays

The plasma concentration of VPA was quantified by fluorescence polarization immunoassay (TDx, Abbott, Rome, Italy) on a TDx apparatus (Abbott). Calibration curves (0.7–75 μg/ml) were prepared by spiking known amounts of VPA in rat drug-free plasma. The limits of quantification were 0.7 (g/ml, and day-to-day coefficients of variation were <5%.

The concentrations of CBZ were determined by a specific HPLC assay (Wad, 1984). 100 μl plasma aliquots were extracted in 50 μl of internal standard (heptabarbital, 50 μg/ml in acetonitrile) and 100-μl acetonitrile. After centrifugation, the supernatant was diluted in PBS (2:1), centrifuged, filtrated through Millex-GV 0.22-mm filters (Millipore, Vimodrone MI, Italy), and 20 μl were injected into a Shimadzu HPLC system (Shimadzu Scientific Instrument, Inc, Columbia, MD, U.S.A.). The system consisted in a System Controller SCL-10Avp, a LC-10ADvp solvent delivery module, a LP-10ADvp pump with a FCV-10ALvp Low-Pressure Gradient Flow Control Valve, an on-line DGU-12/DGU-14A Degasser, and a SIL-10ADvp autosampler. The analytical column was a Brownlee Spheri-5 ODS 100 mm $\times$ 4.6 mm, 5 μm (Brownlee Labs, Santa Clara, CA, U.S.A.), heated to 50°C with a T-6300 thermostate (Merck, Darmstadt, Germany) and connected to LaChrom L-7400 (Merck) variable wavelength detector with readings at 210 nm. For the isocratic elution, the mobile phase consisted in sodium monohydrogen phosphate 50 mM (J.T. Baker B.V., Deventer, Holland), adjusted to pH 5.5 with orthophosphoric acid, and acetonitrile (Sigma-Aldrich, Milan, Italy) 80:20 at flow rate of 1.1 ml/min. Calibration curves (0.5–8 μg/ml) were prepared by spiking known amounts of CBZ in drug-free rat plasma. The limits of quantification were 0.1 μg/ml, and day-to-day coefficients of variation were <15%.

Immunohistochemistry

Brain sections were permeabilized for 10 min at room temperature (RT) in PBS—Triton X-100 (0.1%)—goat serum (5%). For BrdU staining, the permeabilization step was followed by a 20 min incubation in PBS—HCl 2N at 45°C. After permeabilization, slices were washed three times in PBS and incubated overnight at RT with primary antibodies diluted in PBS—Triton X-100 (0.1%)—goat serum (5%). The slices were rinsed three times in PBS and incubated for 2 h at RT in appropriate secondary antibodies, used separately for double immunolabeling. After three final washes in PBS, slices were mounted on glass slides and cover-slipped in Gel Mount (Biomedia Burlingame, CA, U.S.A.). The primary antibodies were as follows: mouse anti-NeuN (1:1,000, Chemicon, Temecula, CA, U.S.A.), rat anti-BrdU (1:50, Harlan Sera-Lab, Gannat, France). The secondary antibodies were as follows: donkey anti-rat conjugated with FITC (1:200) and goat anti-mouse conjugated with Texas Red (1:200) both from Jackson ImmunoResearch (Suffolk, U.K.).

Sections were examined under a Zeiss LSM510 confocal microscope (Le Pecq, France) using 10X and 20X objectives, images were digitized using the built-in software. They were exported as stacks of images, z-projections or orthogonal views in tiff format for quantitative analysis.

Assessment of cell death

An in situ cell death detection kit (Roche Applied Sciences, Meylan, France) was used to detect TUNEL-positive cells, according to the manufacturer's recommended protocol. Briefly, sections were permeabilized for 30 min at RT in PBS—Triton X-100 (0.3%), then rinsed in PBS and pre-incubated with potassium permanganate (0.06%) for 10 min at RT. After additional washes in PBS,

sections were incubated with the TUNEL reaction mixture for 1 h at 37°C.

Sections were examined under on a Nikon Eclipse E800 fluorescence microscope equipped with a Nikon DXM1200 digital camera, using 10X and 20X objectives. Photomicrographs were digitized using the built-in software Nikon ACT-1.

Quantitative analyses

Two different experimenters, blind to the animal's type of treatment, performed the examination of sections and the quantitative analyses. Sections investigated ranged from –2.12 to –6.04 μm from bregma accordingly to the atlas of Paxinos and Watson (1998). Sections (eight sections per animal) were selected randomly, but at least separated one from the others by three sections to minimize the risk of counting the same alterations. Five animals/condition, randomly selected among litter mates born to treated mothers, were analyzed. Cell counting (BrdU+ cells, NeuN+ cells or double labeled cells) was performed on confocal images (Zeiss LSM 510) on selected areas contained within a stack of 30-μm thickness. Images were analyzed with the analysis software ImageJ 1.33d (Wayne Rasband, NIH, Bethesda, MD, U.S.A.) using stereological methods for cells counting. Photomontages and reconstructions were performed with Photoshop 7.0 (Adobe). The normality of the data distribution was checked using SigmaStat (Systat Software, Paris, France), and the effects of treatments were compared using analysis of variance (ANOVA) and the Mann-Whitney as post hoc test.

RESULTS

Developmental alterations induced by prenatal exposure to AEDs

Pregnant rats were treated with VGB, VPA, or CBZ from embryonic (E) day 14 to E19, which correspond to the period of neurogenesis and migration of hippocampal (CA1 region) and neocortical (superficial layers) neurons. This period of treatment was chosen to specifically assess for any potential AEDs-induced alteration of the brain construction consecutive to defects in neurogenesis or neuronal migration. Human fetuses are exposed to AED during the whole duration of gestation including the period of synaptogenesis onset (postnatal in rat pups, end of gestation in humans). However, the aim of the present report was not to evaluate the impact of AEDs in synaptogenesis, a previous report suggested that treatment of postnatal rats with AEDs increase dramatically the rate of apoptotic cells (Bittigau et al., 2002). Consequently pursuing the treatment postnatally would necessarily modify the initial cortical alterations and the destructive changes so induced would make extremely difficult to interpret the effects of such drugs on brain construction.

Plasma drug concentrations during treatment were measured for CBZ (Fig. 1A) and VPA (Fig. 1B) (six animals per treatment). Values obtained for VPA were within the range encountered clinically but values for CBZ were below human clinical levels.

In this article, we also investigated the effect of seizures (kindling) as previous data reported that in addition to adverse effects on maternal and fetal outcome, the occurrence of seizures during gestation might result on cognitive impairment in children born to women with epilepsy (Adab et al., 2004). With this aim we studied the brain of rats born to "kindled" rats that had experienced one generalized convulsive seizure per day during the same gestational period.

Exposure to seizures, VGB and CBZ clearly resulted in reduced maternal weight gain (Fig. 2) and smaller litter size (12 ± 0.5 litter pups in vehicle-treated controls vs. 6.7 ± 0.5, 6.0 ± 2.5, and 7.2 ± 0.5, respectively; $p < 0.05$). VPA had no effect on litter size (12.1 ± 0.7) and maternal weight. Correlation analysis of body weight gain at E19 and litter size presented positive correlation coefficients and p-values below 0.05 (Spearman rank order correlation). These data suggest that exposure during gestation to seizures VGB, and CBZ resulted on fetal lethality.

Conventional histology (Nissl staining) and NeuN (a specific neuronal marker) immunohistochemistry were performed in adult offspring at postnatal (P) day 30. A significantly increased number of hippocampal and cortical dysplasias were observed in rats born to VGB- and VPA-treated mothers as compared to controls. On the contrary, no microdysplasias or other cortical pathological abnormalities were observed in offspring of rats treated with CBZ or exposed to seizures.

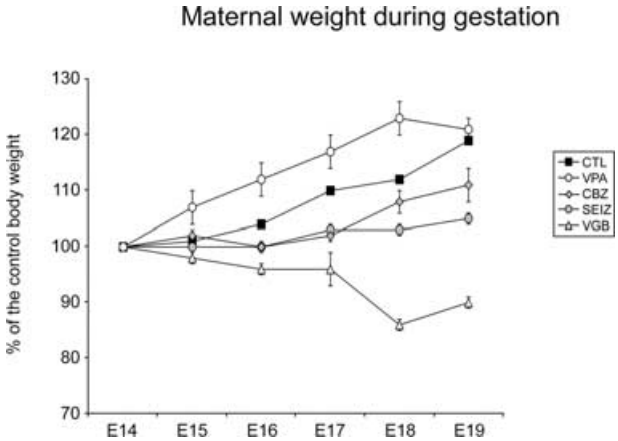

FIG. 2. Maternal weight during gestation. Graph illustrating the maternal weight (percentages ± SEM) monitored during gestation (from E14 to E19) at birth, for each treatment. Note the lower gain of body weight and the smaller litter size after exposure to VGB, CBZ, or seizures (SEIZ). By contrast VPA did not reduce these parameters. The number of pregnant rats and litter analyzed were eight for CBZ, eight for VGB, eight for VPA, four for kindled rats and eight for controls (CTL).

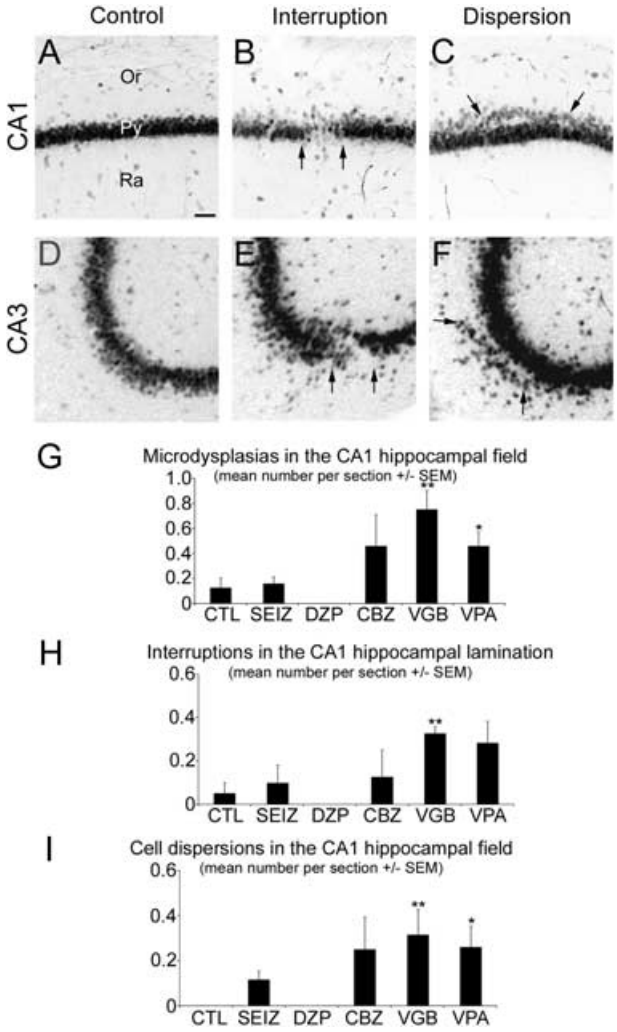

FIG. 3. Fetal exposure to VGB and VPA causes microdysplasias in the CA1 hippocampal field. NeuN immunostainings of CA1 (**A–C**) and CA3 (**D–F**) hippocampal fields, as observed in sections from a P30 rat born to a vehicle-treated mother (**A–D**), and different examples of hippocampal microdysplasias, as observed in animals exposed prenatally to VGB: interruptions in the CA1 (**B**) or CA3 (**E**) lamination and cell dispersions associated with a double layer in the CA1 (**E**) and CA3 (**F**) fields. Arrows point out the extent of the alterations. Scale bar, 50 μm. The histograms illustrate the mean number ($\pm$SEM) per section of 1) any type of microdysplasias in the CA1 hippocampal field (**G**), 2) interruptions in the CA1 hippocampal lamination (**H**) and 3) cell dispersions in the CA1 hippocampal field (**I**) as a function of the treatment received by pregnant animals. Five animals and eight separated sections per animal were quantified. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ as compared with control rats.

In the hippocampus, two kinds of alterations were observed (Fig. 3): (a) interruptions in the Ammon's horn lamination and (b) cell dispersions.

Interruptions in the lamination were manifested by an interruption (Figs. 3B, E) of the continuity of the smooth pyramidal cell layer present in control rats (Figs. 3A, D). Interruptions were mainly observed in rats exposed prenatally to VGB (Fig. 3H) and concerned both the CA1 and CA3 (supplemental Fig. 1) pyramidal layers but not the granule layer, which is formed after birth and was not affected by prenatal AED exposure. These alterations were scored accordingly to their severity and classified into interruption subtypes (see Fig. 4) as mild (the interruption diameter ranged from 10 to 20 μm, Fig. 4A), intermediate (the interruption diameter ranged from 30 to 60 μm, Fig. 4B) and severe (the interruption diameter was 70 μm or larger, Fig. 4C). These interruptions were frequently associated with cell dispersions. The most severe degrees of alterations were found in VGB- and VPA-exposed offspring (Fig. 4D).

Cell dispersions were manifested by a concentration of neurons outside the main pyramidal layer and could give rise in some cases to a double layer composed mainly of ectopic pyramidal neurons (Figs. 3C, F). Dispersions were observed mainly in the CA1 region in rats exposed prenatally to VGB or VPA (Fig. 3I). These alterations were scored accordingly to their severity and classified into cell dispersion subtypes (see Fig. 4) as mild (scattered neurons present within the outer third of the stratum oriens, Fig. 4E), intermediate (dispersed neurons tend to aggregate and form a double layer, Fig. 4F) and severe (high neuronal dispersion associated with pyramidal cell layer ondulations, Fig. 4G). The most severe degrees of alterations were found in VGB- and VPA-exposed offspring (Fig. 4H). Though CBZ exposure did not significantly affect the number of hippocampal interruptions and dispersions in offspring, there was a nonstatistically significant trend for these alterations to increase in CBZ-exposed animals (Fig. 3G), even though these were of a minor degree (Fig. 4). Similarly, prenatal exposure to maternal seizures did not cause an increase in number of interruptions, but there was a nonstatistically significant trend for seizure-exposed offspring to show more dispersions that were essentially of a mild degree, as compared with controls (Figs. 3 and 4).

The two types of hippocampal alterations frequently coexisted. In particular, 69 $\pm$ 5% of VPA-induced cell dispersions were associated with layer interruptions ($p < 0.05$) (see also supplemental Fig. 1).

In the somatosensory cortex, alterations in the layering were observed in the offspring of rats treated with VGB and VPA during pregnancy (Fig. 5C) but not in the offspring of control rats or rats exposed to CBZ or seizures. VGB- and VPA-exposed rats showed massive interruptions in the neocortical lamination, affecting the most superficial cortical layers (layer 2–3), associated with an important cellular depletion both locally and in deeper layers (Figs. 5A, B).

Prenatal-exposure to AEDs increases the incidence of neuronal cell death

To determine whether dysplasias are associated with neurodegeneration, dying neurons were labeled by using the TUNEL technique in slices from P0 rats exposed

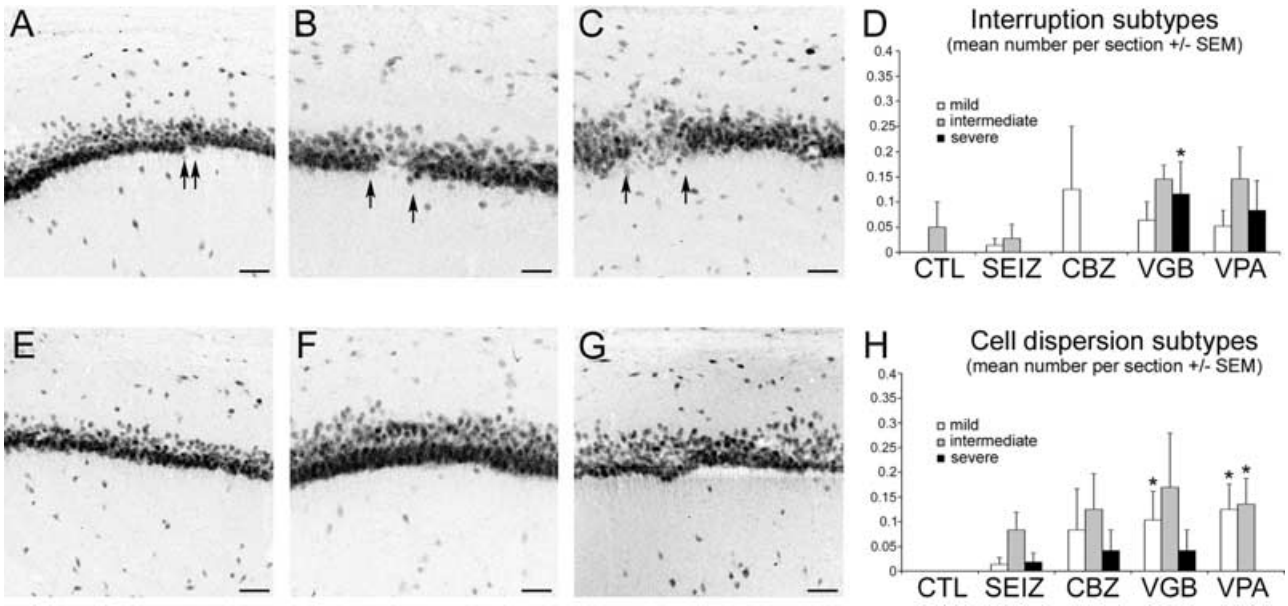

FIG. 4. Severity of alterations induced by fetal exposure to VGB and VPA in the CA1 hippocampal field. A–C. NeuN immunostainings from P30 rats born to VGB- (AB) or VPA (C) treated mothers, illustrating layer interruptions (arrows) associated with cell dispersions. The extent of the interruptions was classified as: (**A**) mild (the interruption diameter ranged from 10 to 20 μm), (**B**) intermediate (the interruption diameter was ranging from 30 to 60 μm), and (**C**) severe (the interruption diameter was 70 μm or larger). Scale bars, 50 μm. (**D**) Histogram illustrating the mean number of each interruption subtypes detected in the CA1 pyramidal layer (±SEM) as a function of the treatment received by pregnant animals. $^*p < 0.05$. Five animals and eight separated sections per animal/condition were quantified. E–G. NeuN immunostainings from P30 rats born to *VGB*- (E, G) or VPA- (F) treated mothers, illustrating layer dispersions. The extent of the dispersions was classified as: (**E**) mild dispersions, with cells scattered within the outer third of the stratum oriens. (**F**) Intermediate dispersions with cells forming a double layer; (**G**) Severe dispersions, with large dispersion associated with undulations of the layer. Scale bars, 50 μm. (**H**) Histogram illustrating the mean number of each cell dispersion subtypes detected in the CA1 pyramidal layer (±SEM) as a function of the treatment received by pregnant animals. $^*p < 0.05$. Five animals and eight separated sections per animal/condition were quantified.

prenatally to VGB or VPA, as well as control rats and rats exposed prenatally to seizures (Fig. 6). Prenatal exposure to VGB and VPA caused a significant increase in the density of TUNEL+ cells in comparison with control animals (Figs. 6A–C). Interestingly, animals exposed prenatally to maternal seizures were not different from control animals in terms of neurodegeneration, indicating that maternal seizures, at least during the last week of gestation, did not induce cell death in the fetal brain. These data are in line with a previous study showing a proapoptotic effect of AEDs, including VGB and VPA, when administered postnatally to rodents, i.e., once neurons have completed their migration and undergo a period of intense synaptogenesis (Bittigau et al., 2002). However, Bittigau et al. (2002) did not report any microdysplasia, supporting the notion that cell death by itself is not the major contributor to the genesis of the alterations reported here and that the cortical and hippocampal dysplasias observed in our study mostly result from other mechanisms.

Prenatal exposure to AEDs perturbs neuronal migration

Because alterations of neuronal proliferation and migration may lead to a variety of developmental disorders, the effects of AEDs and seizures on these parameters was also investigated. Pregnant females were injected with the S-phase marker BrdU at E15 and the number of labeled neurons was counted at P30. The total number of BrdU+ cells detected in hippocampal sections was not modified upon exposure to any AED or maternal seizures (data not shown), indicating that the proliferation at E15 was unaffected by AEDs or seizures. Though this protocol does not allow determining eventual effects on proliferating cells over time, they suggest that neuronal proliferation is not severely perturbed by AEDs.

The examination of the distribution of BrdU+ cells, double-labeled with the neuronal marker NeuN, revealed that in control animals the neurons generated at E15 accumulated mainly in the stratum pyramidale in adulthood, whereas in animals exposed prenatally to VGB and VPA, these neurons were significantly increased in the stratum oriens, with a parallel reduction in the percentage of cells within the strata pyramidale and radiatum (Figs. 7A–C). This suggests that in VGB- and VPA-exposed animals pyramidal neurons fail to migrate correctly and remain in an ectopic position in the adult hippocampus. Similarly, the total number of neurons (NeuN+ cells) was also significantly increased in the stratum oriens and in the dispersion area, as compared with control animals (Figs. 7D). These results are in line with those obtained

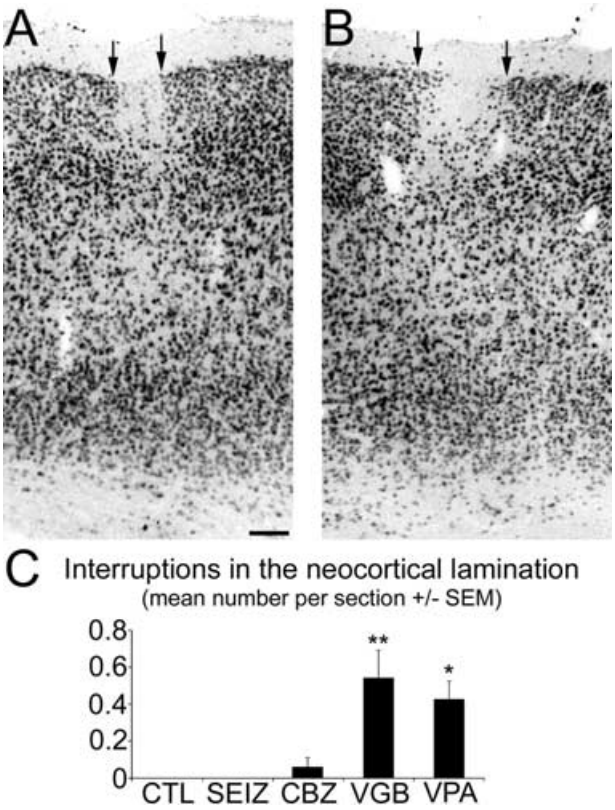

FIG. 5. Fetal exposure to VGB and VPA causes interruptions in the neocortical lamination. (**A–B**) NeuN immunostainings of the somatosensory cortex illustrating two examples of massive interruptions in the neocortical lamination (indicated by arrows), affecting the most superficial cortical layers (layer 2–3), associated with an important cellular depletion at the level of the lesion and in the corresponding deeper layers. Scale bar, 50 μm. (**C**) Histogram illustrating the mean number per section (±SEM), of interruptions (larger than 40 μm) in the neocortical lamination, as a function of the treatment received by pregnant animals. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with controls.

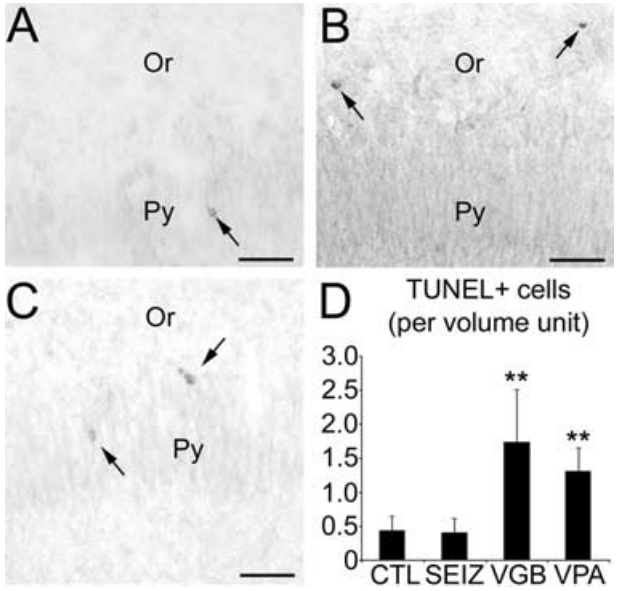

FIG. 6. Fetal exposure to VGB and VPA causes cell death. (**A–C**) Hippocampal sections stained with TUNEL from P0 rats born to mothers treated with vehicle (a), *VGB* (b), or *VPA* (c). TUNEL+ cells are indicated with arrows. Py: stratum pyramidale, Ra: stratum radiatum. Scale bars: 50 μm. (**D**) Histograms illustrating the mean number (±SEM) of TUNEL+ cells per volume unit (6,387,252 μm3) as a function of the treatment received by pregnant animals. $^{**}p < 0.01$.

for the interruption subtypes (Fig. 8), with a significant increase in the number of double-labeled cells (BrdU positive NeuN positive) around the interruption area, as well as into the corresponding strata oriens and radiatum suggesting that neurons accumulated in a dispersion area represent ectopic pyramidal cells whose migration was impaired by AEDs.

DISCUSSION

In this article, we examined the effects of some AED on brain maturation. The choice of VPA, CBZ, and VGB in this investigation was based on a number of considerations: (a) CBZ and VPA are among the AEDs most widely used in the current treatment of epilepsy, and they are the drugs with the largest exposure data in pregnancy registries to date (Perucca, 2005; Morrow et al., 2006); (b) VPA is associated with special concerns with respect to its potential adverse effects on neurodevelopment after prenatal exposure in the clinical setting (Gaily et al., 2004; Vinten et al., 2005; Eriksson et al., 2005; Viinikainen et al., 2006); (c) CBZ, VGB, and VPA allow exploration of different mechanisms of drug action, e.g., blockade of voltage dependent sodium channels for CBZ (White et al., 1999), selective increase in brain GABA levels for VGB (Grant and Heel, 1991) and potentiation of GABAergic transmission coupled with additional modes of action for VPA (Loscher, 2002). The dosages selected were not greatly in excess of those used clinically and, at least in the case of VPA and CBZ, resulted, respectively, in plasma drug levels that were within the clinically occurring range or below. There were also limitations in our study. In particular, plasma concentration profiles after i.p. administration in rats do not mimic closely those observed after orally administered therapeutic doses in the clinical setting. In the case of VPA, in particular, the administration schedule used resulted in marked swings between peak and trough concentrations, a potentially important feature in view of the observation that high peaks in plasma VPA concentrations are critical in adversely affecting embryonic central nervous system development in rodents (Nau et al., 1991). Another limitation was the fact that only one dose was used for each drug, a decision prompted by the complex and labor-intensive nature of the methodology used. In addition, the window time of exposure to AEDs was shorter in our paradigm than in clinal conditions so that the deleterious effects reported here are likely to be more prominent in the latter.

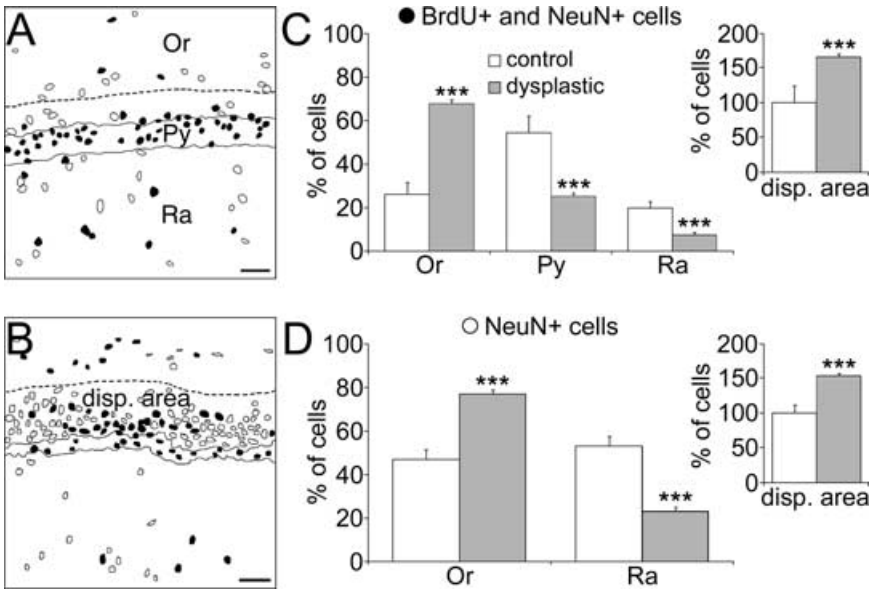

FIG. 7. Cell dispersions caused by fetal exposure to VGB and VPA are linked to migration defects. Reconstructed hippocampal sections illustrating a control CA1 field (**A**) and a dysplastic CA1 field (**B**) with a severe cell dispersion associated with pyramidal cell layer undulation. A single dose of BrdU was injected at E15 and animals were scarified at P30. The position of double-labeled NeuN+ and BrdU+ cells (in black) as compared to the whole population of NeuN+ neurons (in white) indicates that the migration of cells generated at E15 was perturbed in animals exposed prenatally to VGB and VPA, because misplaced cells appear to be located into a dispersion area (disp. area, delimited with dashed lines). The border between the pyramidal cell layer and the dispersion area was defined by the loss of typical compaction of pyramidal cell layer and the clear separation of the cell bodies that are no longer in direct contact between them. Hippocampal layers are indicated: stratum oriens (Or), stratum pyramidale (delineated with straight lines, Py), stratum radiatum (Ra). Scale bar: 50 μm. The histograms illustrate the percentage ($\pm$ SEM) of BrdU+ and NeuN+ cells (**C**) and the percentage of NeuN+ cells (**D**) present in the hippocampal strata (main histograms) and within the dispersion area (histograms in insets) in dysplastic as compared with control CA1 fields (***$p < 0.001$). The percentage of cells located within the dispersion area in dysplastic sections was compared to the percentage of cells present within the outer third of the stratum oriens in controls.

While the above limitations should be kept in mind, our findings demonstrate that prenatal exposure to AEDs that increase brain GABA levels (VPA, VGB) during the last week of gestation in rats leads to the formation of dysplasias in the hippocampus and somatosensory cortex. Moreover, the findings suggest that these abnormalities result from neuronal migration defects as well as neuronal cell death.

Although it cannot be excluded that other pharmacological actions contributed to the adverse effects of VGB and VPA in this study, the most likely explanation is that an increase in GABA transmission played a crucial role, as suggested by different lines of evidence. First, unlike VPA, VGB is considered to be selective on producing GABA-mediated effects (Grant and Heel, 1991). Perhaps more importantly, GABA has been shown to provide most of the excitatory drive at an early stage of brain development, both in rodents and in primates (Ben Ari, 2002). GABA signaling modulates a wide range of essential developmental processes (Represa and Ben Ari, 2005) even before synapses are formed, when it acts in a paracrine manner (Demarque et al., 2002) and is able to modulate the migration of pyramidal neuroblasts (Manent et al., 2005).

The lack of comparably deleterious effects of CBZ, a drug that acts primarily by blocking voltage-dependent sodium channels (White, 1999), may be due to the low density of these channels at an early developmental stage (Manent et al., 2005). It should be noted, however, that plasma CBZ concentrations in these experiments were lower than the range found in patients receiving therapeutic doses (see Table 1) and therefore we cannot exclude that greater histological brain abnormalities could occur in animals exposed to higher CBZ concentrations. Therefore, in addition to GABA-acting drugs, drugs acting on other molecular targets, this including voltage-gated ion channels, might have deleterious effects on brain construction. Further studies are required to clarify this point.

The potential clinical applicability of our findings remains to be established, but these observations should be taken into consideration in future studies on neurodevelopmental outcome in children prenatally exposed to AEDs. In fact, there are indications from other animal studies (Vorhees et al., 1988; Phillips and Lockard, 1996) and from clinical observations (Koch et al., 1999; Dean et al., 2002; Adab et al., 2004; Gaily et al., 2004; Hirano et al., 2004; Perucca, 2005; Vinten et al., 2005; Eriksson et al., 2005) that exposure in utero to some AEDs may lead to neurological and/or cognitive alterations. In particular, while CBZ has been regarded as safe (Gaily et al., 2004), VPA has been associated with developmental delay, including lower verbal IQ (Vinten et al., 2005; Eriksson et al., 2005) and higher incidence of additional

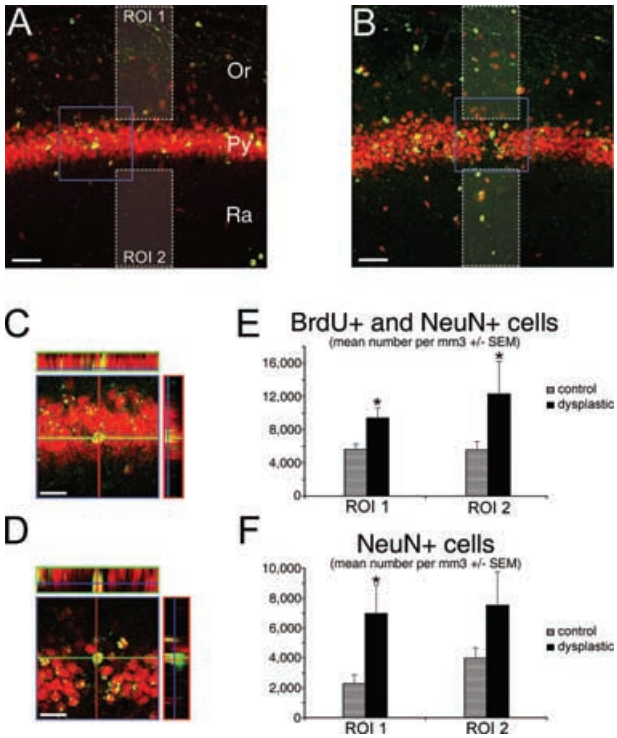

FIG. 8. The interruptions in the CA1 hippocampal lamination caused by fetal exposure to VGB and VPA are linked to migration defects. Confocal images of hippocampal sections (**A–D**) stained with antibodies against NeuN (red) and BrdU (green); green and red channels are merged; scale bars: 50 μm in A–B, 25 μm in C–D. (**A**) Z-projection of three focal planes, from a P30 rat born to a vehicle-treated mother. Two regions of interest (ROI) are indicated: ROI 1 and ROI 2, corresponding to the areas where quantifications have been performed. Hippocampal regions are indicated: stratum oriens (Or), stratum pyramidale (Py) and stratum radiatum (Ra). Note the relatively low density of double-labeled cells into the strata oriens and radiatum, as well as into the ROIs. A higher magnification of the area surrounded by the blue square is shown as an orthogonal view in C. (**B**) Z-projection of four focal planes, from a P30 rat born to a VGB-treated mother (medium interruption associated with cell dispersion). Note the increased number of double-labeled cells around the lesion area, as well as in the ROIs within the corresponding strata oriens and radiatum. A higher magnification of the area surrounded by the blue square is shown as an orthogonal view in D. (**C–D**) Orthogonal views (image stack thickness: 30 μm) of the area surrounded by blue squares in A (C) and B (D), illustrating the colocalization between NeuN (red) and BrdU (green) at the level of the selected cell. (**E**) Histogram illustrating the mean number of BrdU-positive and NeuN-positive cells per mm 3 ($\pm$SEM) present at the level of the ROI indicated in A, in control in comparison with severe interruption subtypes. $^*p < 0.05$ as compared with control. (**F**) Histogram illustrating the mean number of NeuN-positive cells per mm 3 ($\pm$SEM) present at the level of the ROI indicated in A, in control in comparison with severe interruption subtypes. $^*p < 0.05$ as compared with control.

educational needs (Adab et al., 2001; Viinikainen et al., 2006) in prenatally exposed children, even though an influence of confounding factors cannot be excluded (Eriksson et al., 2005; Viinikainen et al., 2006). Several observations suggest that cortical malformations involving the hippocampus and medial temporal cortex are instrumental in mental retardation (Barkovich and Raybaud, 2004). Whether drug-induced subtle cortical and hippocampal dysplasias are involved in causing postnatal cognitive deficits in offspring exposed to VGB, VPA, or other GABAergic drugs during pregnancy should be subject for future investigations, possibly involving careful neuroimaging assessments.

Finally, a possible link between AEDs exposure and epileptogenesis in offspring has been suggested (Phillips and Lockard, 1996; Koch et al., 1999). Though our animals did not apparently displayed spontaneous seizures, it is worth to stress that focal cortical dysplasias are among the most epileptogenic lesions, associated with early onset medically refractory epilepsy (Barkovich and Raybaud, 2004). Therefore, it would be interesting to evaluate whether rats exposed to VGB or VPA during pregnancy have epileptic features or higher susceptibility to convulsant agents.

The use of animal models, such as those we developed in the present study, may also be useful to investigate the mechanisms that lead to cortical dysplasias in order to develop AEDs less deleterious when taken during pregnancy.

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