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Perinatal exposure to GABA-transaminase inhibitor impaired psychomotor function in the developing and adult mouse

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Abstract

Antiepileptic drugs acting through the potentiation of GABA-ergic pathways have harmful effects on brain development. Increased risk of impaired intellectual development was reported in children born to women treated for epilepsy during pregnancy. Here we examined the vulnerability of the developing brain to treatment with one of the new antiepileptic drugs—vigabatrin—during two time periods in newborn mice (postnatal days 1–7 and 4–14) which parallel the third trimester of human embryo brain development. Delayed development of sensory and motor reflexes, reduced mobility in the open field, impaired object recognition and deficient spatial learning and memory were observed independently of the treatment period. On the contrary, specific susceptibility to the age of exposure was detected in various motor functions. A number of morphological correlates may explain these behavioral alterations; a transient increase in CA1 pyramidal cell layer ($P < 0.001$) and decrease in granular cell layer ($P < 0.05$) in hippocampus were detected at postnatal day 7. In addition, a significantly lower cell density was observed in the adult mouse brain in all layers of the M2 cerebral cortex of mice treated during days 4–14, compared to the controls ($P < 0.05$). Our findings demonstrated short- and long-term deleterious effects of vigabatrin treatment and suggest a specific vulnerability of the developing motor system to GABA enhancement during the first postnatal week.

Keywords: Antiepileptic drugs; GABA; Hippocampus; Learning; Memory; Morphogenesis

1. Introduction

Antiepileptic drugs acting through the potentiation of GABA-ergic pathways may have detrimental effects on brain development. Indeed, the increased risk of delayed psychomotor development was described in the earliest reports of the teratogenic effects of antiepileptic drugs (AEDs) (Meadow, 1968; Hanson et al., 1976). Since then, several studies have reported impaired psychomotor development in children born to women treated for epilepsy during pregnancy (Hansen and Lou, 2000). Long-term studies in children exposed to AEDs before birth have demonstrated adverse neurodevelopmental outcomes in 19% of infants, as compared to 3% of control siblings (Dean et al., 2002). In follow-up studies of preschool children, it has been found that AEDs and phenytoin are associated with a decrease in intelligence quotient (IQ) and specific cognitive dysfunctioning (Scolnick et al., 1994). A follow-up study of children in the 6–13 age group showed a connection between prenatal Phenobarbital, a small head circumference and poorer cognitive performance (Van der Pol et al., 1991). Adults exposed prenatally to phenobarbital and phenytoin have also been shown to have a significantly smaller head circumference at birth, significantly increased learning problems and mental retardation in adulthood (Dessens et al., 2000). Antiepileptic drugs have also been shown to be deleterious to neurodevelopmental outcomes when given during infancy. Prolonged treatment with phenobarbital in infants with febrile seizures has been shown to impair cognitive development (Volpe, 2001).

Recent studies in animal models on the effects of short and prolonged exposure to AEDs on the developing brain indicate that modulation of GABA may play an important role in the pathogenesis of brain injury associated with these agents. Vigabatrin (VGB), which inhibits GABA degradation by blocking the enzyme GABA-transaminase, increases the concentration of GABA in the brain (Qume et al., 1995). Treatment with VGB in newborn rats (P14–P26) caused marked white matter damage and resulted in behavioral hyperactivity. Remarkably, after a 2-week recovery period, most of the histological damage had resolved, while

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the behavioral abnormality persisted (Qiao et al., 2000). Hyper-activation of $GABA_A$ receptors (GABA-A R) has behavioral consequences in juvenile rats and subsequently results in a tendency to reduced learning ability (P21) (Nunez et al., 2003b). Lower cell counts within the hippocampus (CA1 and DG) at P21 may partly explain such behavioral changes. Diazepam given during the last week of gestation causes neurobehavioral effects that become apparent in young adult rats (Kellogg, 1998). In newborn rats (P0–P1), significant cell death in the DG and hippocampus occurred a week after treatment with muscimol, a GABA-A R agonist (Nunez et al., 2003a,b).

When given during the first postnatal week, a range of AEDs cause apoptotic neurodegeneration, independent of their mechanism of action (Bittigau et al., 2002). The period of vulnerability to the pro-apoptotic effect of AEDs coincides with the brain growth spurt period, which, in the mouse, spans the first two postnatal weeks of life. In humans, the comparable period begins in the third trimester of gestation and extends to several years after birth.

In the present study, we have examined the vulnerability to VGB at two periods of early postnatal life. We selected to use the new class AED-VGB because it directly affects GABA levels in the cells and in the brain and does not modulate its concentration in the synaptic cleft or the conductance of the $GABA_A$ receptors, as most of the AEDs do.

We demonstrate that perinatal exposure to VGB delays the development of motor and sensory reflexes. In addition, significant reduction in adult offspring exploratory behavior, motor function and learning and memory abilities was detected in the adult offspring in both treatment groups, compared to their controls. The time period of exposure to VGB had a minor effect. Moreover, perinatal exposure to VGB had only a transient effect on hippocampal development, whereas lower cell density was observed in all layers of the cerebral cortex of adult mice treated during postnatal days 4–14, compared to the controls.

2. Materials and methods

2.1. Study design

Balb/C pregnant mice were assigned to one of four groups, as described below, on the day of delivery.

2.1.1. Drug administration

Vigabatrin (Sigma-Aldrich, St. Louis, MO), 50 mg/kg, in a volume of 50 μl (sub-cutaneous), was injected daily to newborns at a regular hour during the light cycle of days P1–P7 (VGB1–7) and P4–P14 (VGB4–14). Control groups were treated with similar volumes of vehicle on the same postnatal days (CT1–7 and CT4–14).

The mouse colony was maintained in a 12:12 hour light/dark schedule; food and water were provided ad libitum. All procedures were performed according to guidelines from the Israeli Council on Animal Care and approved by the Ben-Gurion University of the Negev Animal Care and Use Committee.

2.2. Histology

Offspring at P7, P14, P21 and adults aged 4 months were anesthetized and trans-cardially perfused with paraformaldehyde (PFA) 4%. Seven-day old mice were anesthetized by hypothermia, while older mice were anesthetized by the i.p. administration of Ketamine and Rompun.

Sections from paraformaldehyde-fixed paraffin-embedded tissues were used. Four-micron thick sagittal sections 0.5–0.6 mm from the midline were mounted on saline-coated slides and dried at 37 °C for 48 h. Afterwards, Nissl staining was performed. Images were sampled in an Olympus IX-70 microscope equipped with a SuperCam video camera (Applitec, Israel). The analysis was performed using 'NIH Image' software (Wayne Rasband, NINDS, NIH). The following parameters were measured: cortex width and length, motor cortex cell density and size, corpus callosum width, cerebellum area, cerebellum lobe length, Purkinje cell density, molecular layer thickness, hippocampus area, CA1 pyramidal cell-layer width, and in the dentate gyrus, granular cell-layer width.

2.3. Phenotype aspects examined in newborn mice

Two newborns per litter were tested daily during the first month of life for general phenotypic and morphogenic aspects, such as: body weight, fur growth, day of eyelid opening and teething. The day in which all newborns presented the phenotype was compared. Phenotype and behavioral examination were performed before the daily drug administration.

2.4. Behavioral examination

2.4.1. Reflex development in newborn mice

During the first 2 weeks of the newborn's life, the following reflexes were tested:

Righting reflex was measured in seconds, as the time required for a newborn laying on its back to right itself on all four limbs, the test measures motor function and coordination (Crawley, 1999). *Rotarod* (adjusted for neonates) tests the ability to hold the rotating bar at a rate of four cycles per minute. The duration on the rotarod prior to the falls was recorded. The test measures coordination, motor function and strength (Golan et al., 2004). *Cliff avoidance*—newborns were placed facing a cliff for up to 60 s. If the newborn turned aside to avoid the cliff, the time was recorded; otherwise, a negative response was recorded. The cliff-avoidance reflex developed before eyelid opening, newborns use their whiskers for the sensation of the environment, during this

period they are able to navigate their way without the use of vision.

2.5. Adult mouse behavior

Mouse behaviors in the different tests were videotaped and analyzed off-line with Ethovision Pro. Software. (Noldus, The Netherlands).

Open field task—performance was tested in a circular open field arena: a 65 cm diameter gray wall, 30 cm high, over a period of 5 min. The following variables were observed: rearing (frequency), center/perimeter ratio, distance moved, walking velocity, frequency of entries to the center of the field and general pattern of exploration (Henderson, 1967). *Elevated plus maze*—a 'plus maze' with 40 cm long arms and 15 cm high sides, was used to measure the time (seconds) spent in the open versus the closed arms (Lister, 1987). *Footprint pattern* measures ataxia and gait abnormalities. Paws were dipped in nontoxic paint. The mice were then placed at one end of a dark tunnel. The bottom surface of the tunnel was lined with white paper. The mice walked down the tunnel leaving their footprints. Stride distance, width, overlap between forelimbs and hind limbs, and the number of stops were measured. *Balance beam* measures motor coordination and balance. A beam, 8 mm in diameter and 70 cm long, was placed horizontally and elevated. Enclosed escape boxes were placed at the edges of the beam. The mice were placed in the center of the beam. The times required to reach the box and the duration on the beam were measured (Crawley, 1999). *Grip strength test*—the grip strength meter was positioned horizontally and mice were held by the tail and lowered toward the apparatus. Mice were allowed to grasp the smooth, metal, triangular pull bar (forelimbs only) and were then pulled backward in the horizontal plane. The force applied to the bar at the moment the grasp is released is recorded as the peak tension (gr). This test was repeated four consecutive times within the same session and the average was recorded as the grip strength for that animal. *Morris water maze*—mice were trained to swim to a hidden platform in the water maze (Morris, 1984). The maze consisted of a 150 cm diameter, 40 cm high, circular pool filled with powdered milk water and maintained at 25–26 °C. The 10 cm diameter platform was 0.5–1 cm below the surface of the water. The pool was placed in the center of an enclosure with white walls and a colored geometric figure on each wall (triangle, rhombus, circle and rectangle) that served as spatial cues. One day before the training, the mice were placed on the platform for 10 s, if they fell they were replaced on the platform. Three- to four-month-old mice were tested in a blind analysis and were randomly assigned for training to find the platform in a given quadrant. For six consecutive days, each mouse was placed in the pool four times, starting in a random order of direction: north, south, east or west with 30 min intervals between trials. After locating the platform, the mice were dried and returned to their cages. If, after 60 s in the pool, the mice could not locate the platform, the trial was stopped and the escape time was recorded as 61 s. *Probe test*: at the seventh day, the platform was removed from the pool. The mice were placed in the pool at a location opposite to the platform and were allowed to swim for 60 s. The time spent swimming in each quadrant was recorded. The amount of time spent swimming in the quadrant where the platform had been located was recorded as another index of spatial learning. The mice were housed in individual cages on a 12-h light/dark cycle and were tested between 16:00–20:00 h daily. *Object recognition test* (Malleret et al., 2001): during the training trial, the mice were placed in a novel environment (55 cm diameter, 20 cm high). Two (of three possible) plastic toys (between 5 and 7 cm) that vary in shape and texture were placed in specific locations in the environment 30 cm apart. Two different combinations of object pairs were used. The mice were allowed to explore the environment and objects freely for 15 min and then were returned to their individual home cages. After 24 h, the mice were brought back to the environment with two objects in the same locations, but one of the familiar objects was replaced with a novel object. The mice were then allowed to explore both objects again freely for 15 min. The number of seconds spent exploring each individual object and the frequency of interactions with the objects were analyzed. A mouse was considered to be exploring the object when it was at a distance of 2.5 cm or less from the object. The total number of seconds spent exploring each object during the testing phase was calculated and the time the mouse spent exploring the novel object was divided by the amount of time spent exploring the novel and familiar objects. A ratio greater than 0.5 indicates a preference for the novel object during testing, whereas a ratio lower than 0.5 indicates a preference for the familiar object.

3. Results

3.1. Development of morphogenic phenotype and reflexes

VGB treatment during P4–14, but not P1–7, significantly delayed eyelid opening and fur growth in newborns as shown in Table 1. Body weight was differentially effected in both groups during the first month of newborns' life. In the second month and later, body weight was reduced in offspring of both VGB1–7 and VGB4–14, compared to their controls (Fig. 1A).

The development of the sensory reflex, cliff avoidance, is depicted in Fig. 1B. Cliff avoidance reflex developed later in both treated groups, as demonstrated by slower response time, when newborns faced a cliff. These differences were observed mainly before P9. At P9 all the groups presented a similar response time.

The effect of perinatal VGB treatment on the development of motor reflexes depended on the treatment period. VGB treatment during P1–P7 significantly delayed the development of righting reflex in newborns, as illustrated in Fig. 1C(left); newborns from the control group righted

Table 1
Development of morphogenic parameters in newborns

Treatment	Age of treatment			
	1–7		4–14	
	Control	VGB	Control	VGB
Eyelid opening	14.15 ± 0.2 ($n = 13$)	15 ± 0.01 ($n = 4$)	15.8 ± 0.21 ($n = 24$)	15.6 ± 0.23* ($n = 31$)
Teething	6.18 ± 0.2 ($n = 13$)	6.31 ± 0.3 ($n = 17$)	8.08 ± 0.3 ($n = 52$)	10.56 ± 0.2 ($n = 31$)
Fur growth	7.43 ± 0.2 ($n = 12$)	7.67 ± 0.14 ($n = 14$)	10.1 ± 0.19 ($n = 20$)	8.65 ± 0.26* ($n = 37$)

The day on which newborns achieved the criteria (mean ± S.E.M.). At each day during the first two weeks two newborns per mother were tested, n in the table includes only the animals which achieved the criteria..

* $P < 0.0001$.

themselves in a time shorter than 1 s at P7, while the VGB1–7 newborns could right themselves in a similar time 4 days later, at P11. In contrast, no difference was observed in the development of the righting reflex between offspring of VGB4–14 and their control group.

On the other hand, the ability of newborns to grip onto a rotating rod developed normally in the VGB1–7 group, but the VGB4–14 group was delayed in the development of this skill, compared to the control group (Fig. 1D). At the age P14, the VGB4–14 newborns could grip the rod when rotated 180°, compared to 360° in the control group.

Thus, the perinatal exposure to VGB, independent of the period of treatment, had a long-term influence on newborns' body weight and a short-term influence on the development of cliff avoidance reflex. The consequences of VGB treatment on the development of motor reflexes depended on the time period of exposure to the drug.

3.2. Exploration and anxiety-related behavior

Exploration in the open field was examined in all the groups of young mice, aged 7 weeks and in the adult mice, aged 4 months. At the age of 7 weeks no difference between VGB1–7, VGB4–14 and their control groups was found in the distance moved, walking velocity, rearing and the ratio between the time spent in the perimeter versus the time spent in the center of the arena, as demonstrated in Fig. 2A and B. However, at the age of 4 months, both treated groups were less mobile than their controls. This was reflected by a shorter distance moved in the arena and a slower walking velocity. In Fig. 2C and D, the maximal distance covered in one path in the whole arena showed a significantly lower value for VGB treated mice ($P < 0.01$). A tendency towards a slower walking velocity was also measured in the VGB4–14 group ($P < 0.05$), while VGB1–7 had a significantly slower walking velocity, compared to the control group ($P < 0.1$). VGB did not effect tigmotaxis, as indicated by a similar distance to the border of the arena in all groups and a similar ratio of time that the mice explored the perimeter of the field versus the center of the field. This may suggest that the VGB treatment did not modify mouse anxiety, but rather influenced mouse mobility in the arena. To further examine a possible effect on mouse fear, we tested the VGB-treated offspring and the corresponding controls in the elevated plus maze in adulthood. Mice from VGB1–7 and VGB4–14 spent a similar time period in the dark areas, compared to their controls. A slight difference was observed in the VGB1–7 group, which showed a trend for a shorter distance covered in one path within the dark areas (3.2 ± 0.24 cm versus 4.03 ± 0.3 cm in the control, $P < 0.1$), but no difference was observed in the time during which mice explored the dark areas (292.44 ± 3.52 s, in VGB1–7 mice and 296.1 ± 2.97 s, in the controls). Although there were no quantitative differences, the movement pattern showed differences between VGB4–14 mice and controls, but not between VGB1–7 and their controls. In the control group, 60% moved preferentially in the dark arms (Fig. 3A and D), compared to 33.3% in the VGB4–14 group.

3.3. Motor function and grip strength

The patterns of walking and ataxia were analyzed in 4-month-old mice by analyzing their footprints. A significant increase in stride width was observed in VGB1–7 hind limbs 27.8 ± 0.74 mm, compared to 22.3 ± 1.05 ($P < 0.001$) in the controls. The same trend was observed in forelimbs; 17.1 ± 0.8 mm in the VGB1–7 group, compared to 14.9 ± 0.9 in the control group (Fig. 4A). Similar stride length was observed in VGB1–7 and control offspring, when measured for forelimbs and hind limbs in both the left and right feet. In contrast, no differences between the VGB4–14 and their control group were observed in their footprint analysis. The number of stops during the test was similar in all the groups (Fig. 4B). Additional examination of motor function, balance and coordination, was performed by testing the escape time on the balance beam. Mice in the VGB1–7 group reached the escape box after a longer period (53.8 ± 6.1 s), compared to the control group (33.7 ± 8.8 s, $P = 0.07$), whereas a similar escape time was observed in the VGB4–14 and the control group (26.3 ± 7.7 and 26 ± 7.1 s, respectively, Fig. 4C and D). Another aspect of motor function examined was grip strength; reduced grip strength was measured in the VGB1–7 group, compared to the controls (89.9 ± 4.9, and 106.1 ± 5.4 g, respectively, $P < 0.05$), as shown in

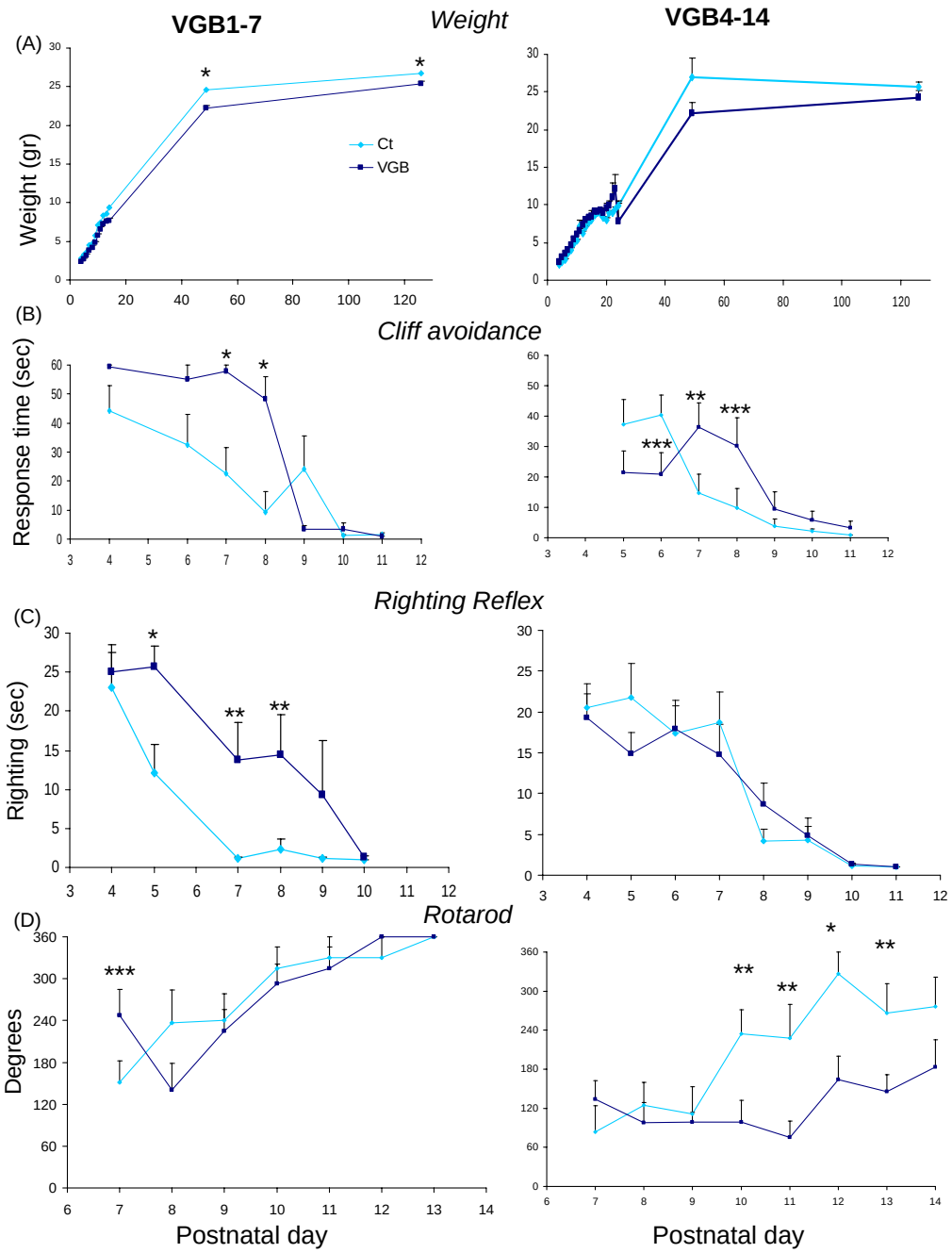

Fig. 1. Effects of perinatal VGB treatment on newborn development. (A) VGB1–7 and VGB4–14 reduced adult mice body weight. *n*(Ct1–7 and 4–14) = 4–8, *n*(VGB1–7 and 4–14) = 5–8. (B) The development of the cliff avoidance reflex was delayed in both treatment groups. (C) Righting reflex was examined until all newborns righted themselves in less than a second. VGB1–7, but not VGB4–14, showed a delayed development of the righting reflex. (D) The ability to grip a rotating rod was measured until all newborns in the control groups could hold onto the rod for a full cycle (360°) on three consecutive days; this ability was reduced in VGB4–14. For B–D: *n*(Ct1-7) = 6–8, *n*(VGB1–7) = 6–8, *n*(Ct4–14) = 6–14, *n*(VGB4–14) = 10–14; $^{*}P = 0.01$, $^{**}P = 0.05$, $^{***}P = 0.1$.

Fig. 4E. Similar grip strength was measured in offspring of the VGB4–14 group and offspring of the control group (77.8 ± 9.1, and 88.2 ± 4.2 g, respectively, Fig. 4F).

Overall, perinatal treatment with VGB during P1–P7 had long-term consequences on various aspects of motor function in the adult offspring. Similar treatment during P4–P14 did not interfere with the development of proper motor function.

3.4. Learning and memory

The ability of mice to discriminate between a novel and a familiar object was examined in the object recognition task. On the first day of the test, when the mice were introduced to a novel environment containing two novel objects, the ratio of the duration of exploration: object 1/(object 1 + object 2) was 0.47 ± 0.04 for the control group (CT1–7). On the

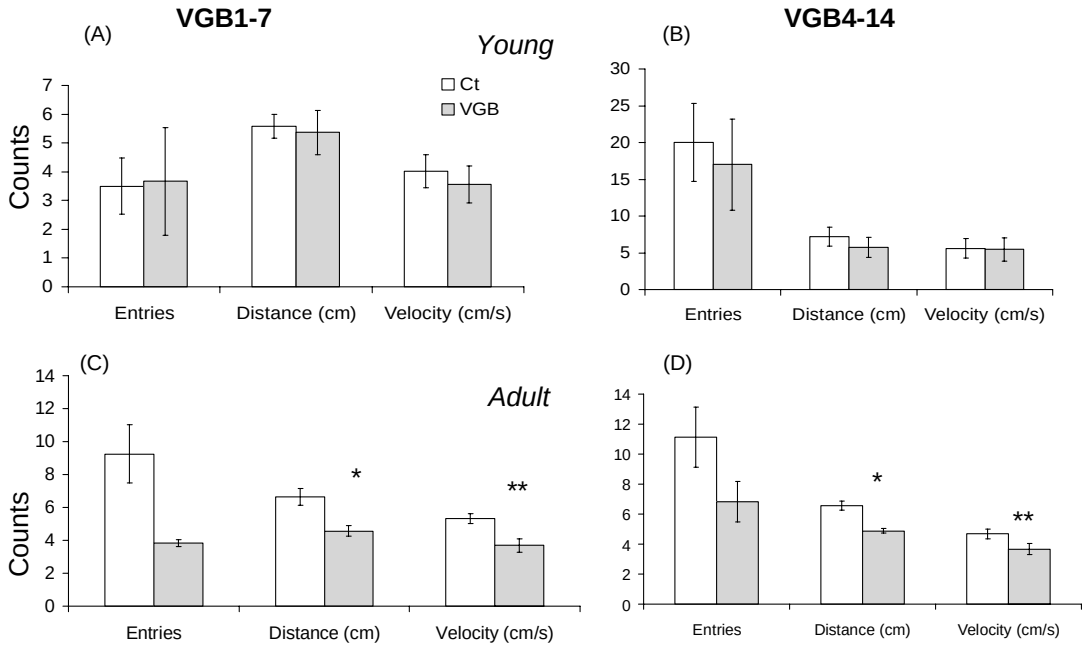

Fig. 2. Effects of perinatal VGB exposure on open field exploration. Mouse exploration in the open field was examined in young, 7-week-old mice (A, B) and in adult 4-month-old mice (C, D). The number of entries to the center of the field (determined as an area with a 30 cm diameter at a distance of 15 cm from the arena walls), maximal distance crossed in a single path and the movement velocities are shown. For A and B: n(Ct1–7) = 8, n(VGB1–7) = 6, n(Ct4–14) = 4, n(VGB4–14) = 4. For C and D: n(Ct1–7) = 7, n(VGB1–7) = 5, n(Ct4–14) = 8, n(VGB4–14) = 5. $^{*}P = 0.01$, $^{***}P = 0.1$.

second testing day, when object 1 was replaced by object 3, the mice showed a preference for a longer exploration time of the novel object (object 3), compared to the duration of exploration of the familiar object (object 2), resulting in a ratio of 0.62±0.04 ($P < 0.05$). The offspring of the VGB1–7 group had a similar exploration ratio on both experimental days (0.61 ± 0.04 and 0.62 ± 0.04, for the first and second day, respectively; $P = 0.53$), indicating that these mice did not distinguish between the novel and the familiar object. A similar result was observed when the VGB4–14 mice and the controls were tested; the VGB4–14 presented a ratio of 0.59 ± 0.04 on the first day and of 0.6 ± 0.06 on the second day ($P = 0.8$), whereas the control group had increased the ratio from 0.51 ± 0.04 to 0.69 ± 0.03 ($P < 0.005$), respectively. The percent of change for all the groups is presented in Fig. 5A and B.

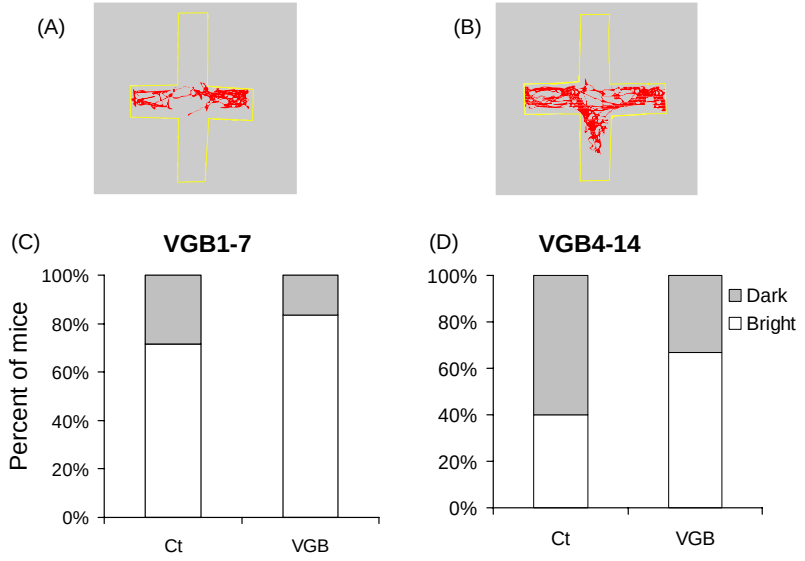

Fig. 3. The effect of GABA on the pattern of exploration in the elevated plus maze. Two patterns of exploration were defined: (A) preference for exploration in the dark arms, (B) no preference for exploration in the dark or bright arms. Classification for either pattern was performed by an observer blind to mice groups. The percent of mice which behave according to each pattern is shown in C and D. n(Ct1–7) = 7, n(VGB1–7) = 6, n(Ct4–14) = 5, n(VGB4–14) = 6.

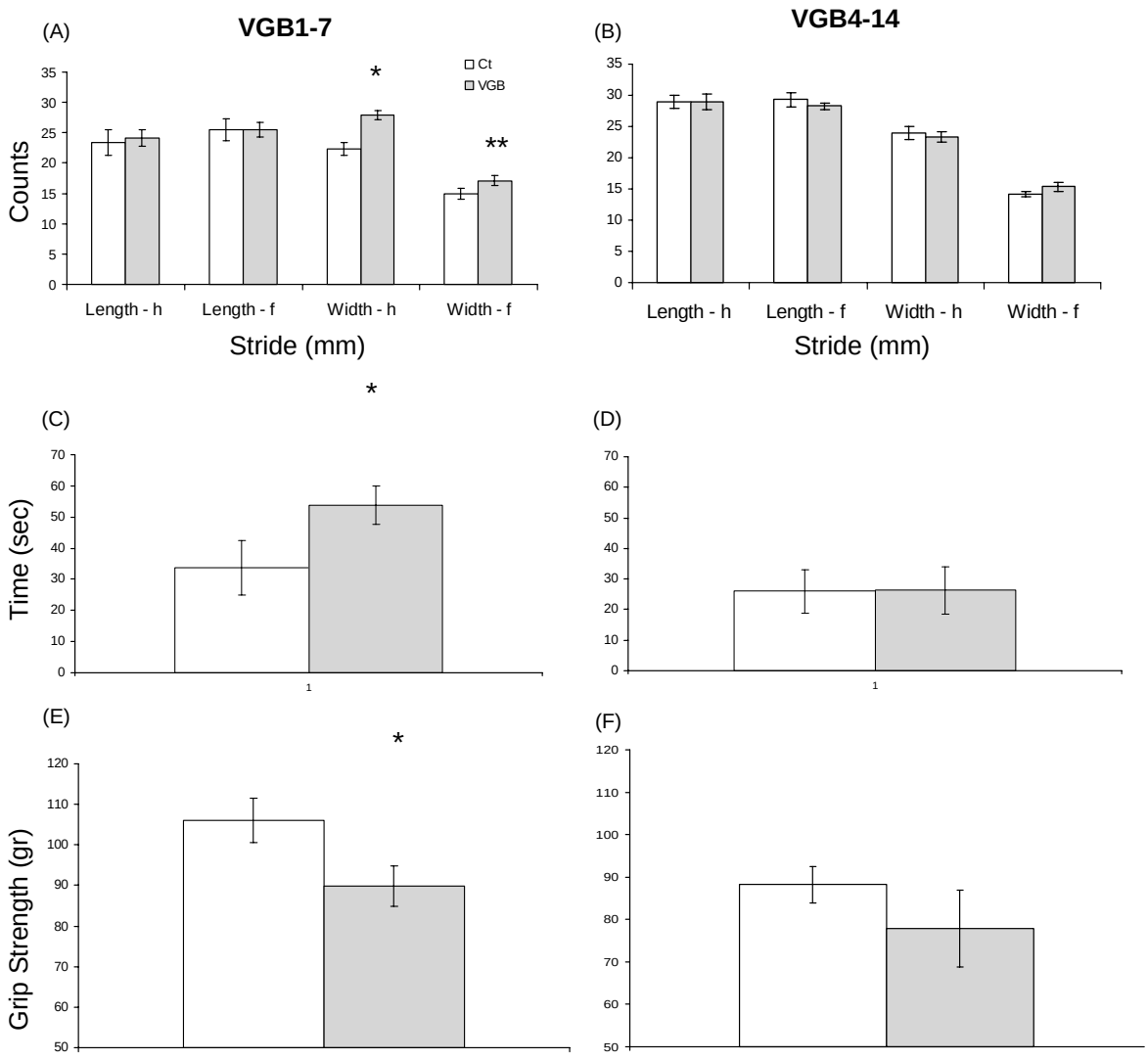

Fig. 4. Motor functions were differentially modified by the time period of the treatment. Walking patterns examined in adult mice of VGB1–7 and VGB4–14, show specific effects in the VGB1–7 group. Stride length for forelimbs and hind limbs, and stride width are presented (A, B), n(Ct1–7) = 6, n(VGB1–7) = 4, n(Ct4–14) = 5, n(VGB4–14) = 6. The time required to reach an escape box in the balance beam test is presented for both groups (C,D) n(Ct1–7) = 7, n(VGB1–7) = 6, n(Ct4–14) = 6, n(VGB4–14) = 7. Grip strength as measured in a grip strength apparatus (an average of four tests per animal) is presented (E, F). n(Ct1–7) = 8, n(VGB1–7) = 6, n(Ct4–14) = 6, n(VGB4–14) = 5. $^{*}P < 0.05$.

In order to confirm the possibility that perinatal exposure to VGB impaired learning and memory abilities, mice were tested in the Morris water maze. Mice from the control and VGB4–14 groups learned to find a hidden platform using spatial cues. Mice from both groups improved their speed in finding the platform during the training days, however, mice from the VGB4–14 group improved their performance during the first four days of training and then during the following days their speed remained constant. This resulted in a significant difference between the control and VGB4–14 group on the fifth and sixth days of training. The recollection of platform location was examined 24 h after the last training session. Mice from the control group searched the quarter where the platform had been placed previously for 28.5 ± 3.7 s, compared to 14 ± 3.5 ($P = 0.01$) in the VGB4–14 group.

These results indicate that the VGB4–14 group did not remember the platform location.

In summary, perinatal exposure to VGB caused long lasting impairment of recognition and spatial memory in adult mice.

3.5. Brain morphogenesis

In order to find whether perinatal exposure to VGB induced changes in morphogenesis, which may explain the behavioral outcome described above, several brain regions were examined during development and in adult mice; cerebellum, hippocampus and motor cortex.

No differences were observed in the gross measurements of cerebral cortex width (M2), cerebellum area and hippocampal area between the control and VGB1–14 groups

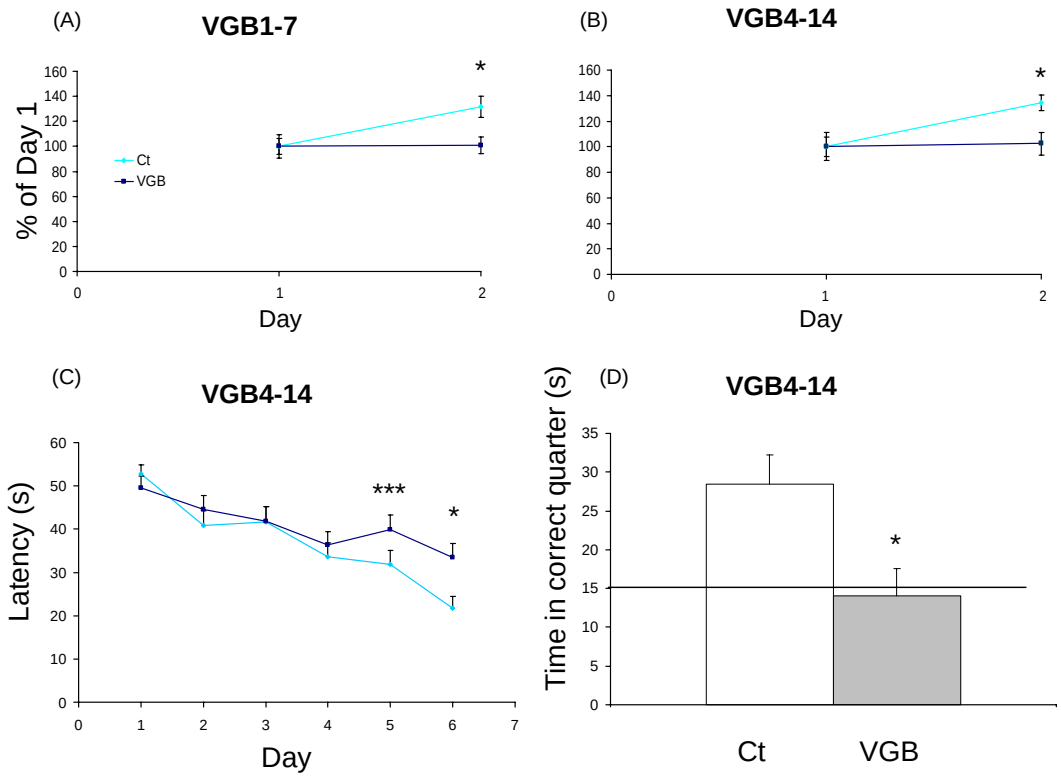

Fig. 5. Learning and memory abilities were reduced due to the perinatal exposure to VGB. The ability of mice to discriminate between novel and familiar objects in the object recognition test was presented as the percent of change in the ratio: novel/(novel + familiar) on the second day as a percent of the first day (A, B) n(Ct1–7) = 8, n(VGB1–7) = 6, n(Ct4–14) = 9, n(VGB4–14) = 7, $P < 0.05$. Spatial learning in the Morris water maze was examined in the VGB4–14 group. The average time required to find the platform on each training day is presented (C). The time the mice searched in the quarter where the platform was located during training sessions was measured 24 h after the last training day. The horizontal line represents their random searching (15 s in each quarter) (D). For C and D: n(Ct4–14) = 12, n(VGB4–14) = 11; $^{*}P = 0.01$, $^{***}P = 0.1$.

(data not shown) at all the ages examined (P7, P14, P21, P120). A detailed analysis of the cerebellum included cerebellar lobule length, Purkinje cell density and molecular layer width (measured in cerebellar lobule 3) and all of which indicated a similarity between the control and VGB4–14 groups. However, in the hippocampus, measurement of CA1 pyramidal cell-layer width and granular cell layer width in the dentate gyrus (DG) showed transient differences at P7 between the groups. A significant increase in the CA1 width and a reduction in the DG width was detected in the VGB4–14 group, compared to their control group. As shown in Fig. 6A and B, these differences were not observed at older ages.

In the cerebral cortex of adult (P120) mice, a detailed analysis of cell size and density indicated that perinatal exposure to VGB did not influence cell size. In layers 2 and 3 of control mice average cell size was $143.1 \pm 6.7\,\mu m^2$ and in the VGB4–14 $149.6 \pm 4.1\,\mu m^2$. In layer 4, cell size was 148.5 ± 0.82 and $144.32 \pm 3.8\,\mu m^2$, and in layers 5 and 6; 148.5 ± 5.2 and $139.16 \pm 1.02\,\mu m^2$, in control and VGB4–14 groups, respectively. However, cell density was significantly reduced in layers 2–4 (Fig. 7). A slight reduction in cell density was observed also in layers 5 and 6 of the VGB4–14 mice, compared to the control group.

Our morphological analysis exposed permanent damage in the cerebral cortex alone as a result of perinatal exposure to VGB during P4–P14. It may be that other brain regions, not examined in the present study, may respond differently to VBG treatment.

4. Discussion

The present study examined the sensitivity of the developing brain to the inhibition of GABA-transaminase during two periods in the early postnatal life of mice which parallel the developmental stages of the last trimester of pregnancy in humans (Dobbing and Sands, 1979). A low therapeutic dose of VGB was used in the current study to avoid robust teratogenic effects of the drug (Abdulrazzaq et al., 1997). In both time periods examined, VGB induced short and long-lasting behavioral modifications. Delay in the development of sensory capability, as examined in the development of cliff avoidance reflex, reduced mobility and impairment of learning and memory were results of VGB treatment, regardless of the times of exposure. On the contrary, a specific defect was observed in the VGB1–7 group in the motor skills of adult mice. A different outcome in

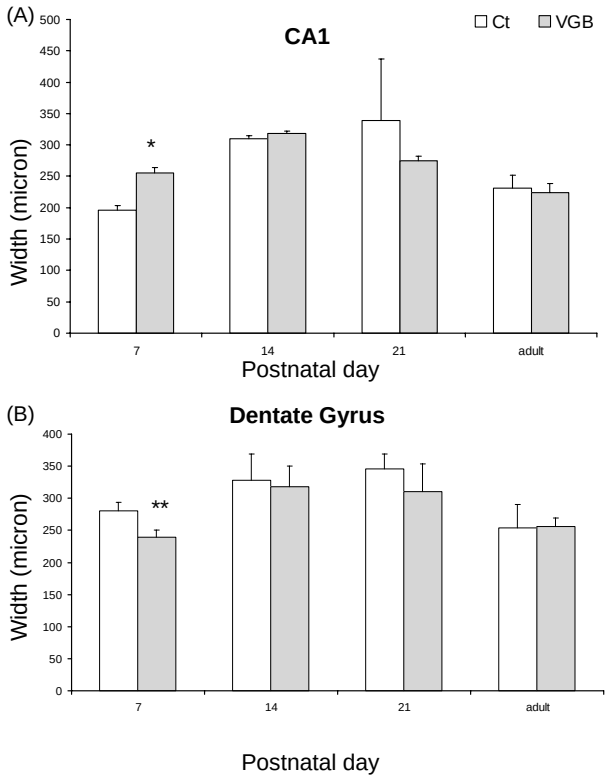

Fig. 6. Effect of perinatal exposure to VGB on hippocampal morphogenesis. The width of the CA1 pyramidal cell layer was examined in P7, P14, P21 and adult mice (P120). Measurements were performed in three areas in three sections separated by 50 μm at sagittal sections 0.5–0.65 mm from the midline (A). Granular layer width in the DG was measured in the same sections (B). $n = 3$–4 animals in each group at each age, $^{*}P = 0.01$, $^{**}P = 0.05$.

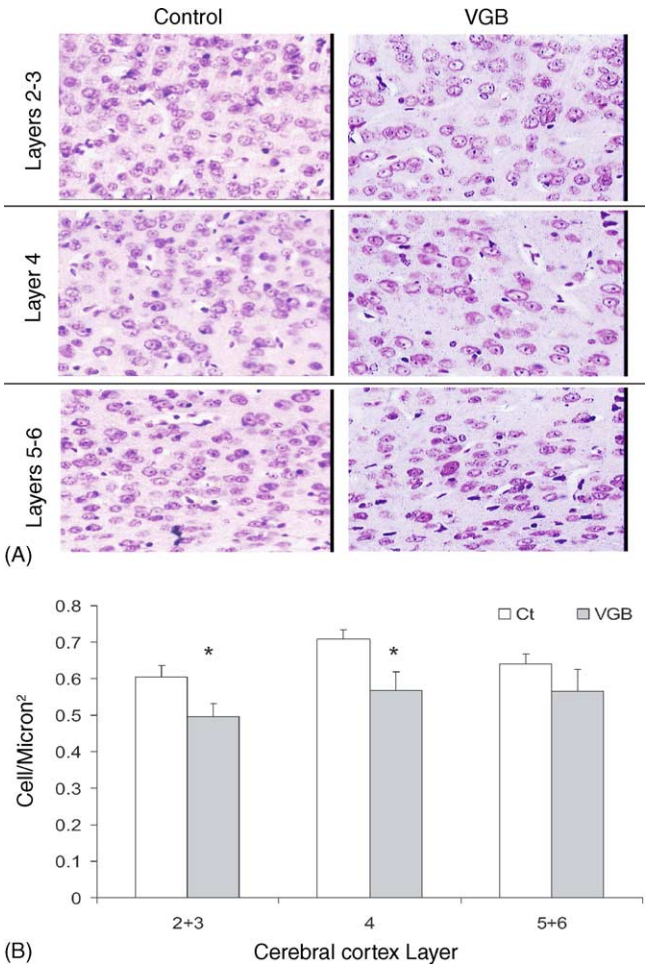

Fig. 7. Perinatal exposure to VGB reduced cell density in the adult M2 cerebral cortex region. (A) An example of Nissl staining in the different cerebral cortex layers is demonstrated. (B) Quantification of cell density in three areas of interest in three sections separated by 50 μm at sagittal sections 0.5–0.65 mm from the midline, reduced cell density in layers 2 + 3 and layer 4 of the M2 cerebral cortex is shown. $n = 3$–4 animals; $^{**}P = 0.05$, $^{***}P = 0.1$.

response to GABA enhancement at various time periods during neurogenesis is expected, since, during ontogeny, the GABAergic system indeed changes remarkably during the late embryonic and early postnatal age (Dupuy and Houser, 1996; Barker et al., 1998). One robust change occurring during the first 2 weeks of life is the expression of the KCC2 transporter for chloride and potassium ions (Rivera et al., 1999; Ludwig et al., 2003; Marty et al., 2002), which results in a switch from depolarizing to hyperpolarizing $GABA_A$-receptor mediated potentials (Rivera et al., 1999). The time periods examined in the present study fit the period in which $GABA_A$ mediated potentials are predominantly depolarizing (P1–P7) and the period when GABA is mainly hyperpolarizing cell membranes (P4–P14). Recently, Quilichini et al. (2003) demonstrated that in the immature brain (P8) VGB was not an effective AED in the prevention of recurrent ictal-like seizures in the hippocampus and neocortex. This finding may suggest that VGB treatment at this age does not enhance inhibition, probably due to the depolarizing effect of GABA during this period.

Given that this transformation from depolarizing GABAergic potentials to hyperpolarizing GABAergic potentials appears gradually and in different intensities in diverse CNS regions (Perrot-Sinal et al., 2003), a major alteration in behavioral outcomes may be detected for particular brain areas. In this regard, the delay in the development of the righting reflex may indicate a specific interaction with axon myelination in addition to the effect of changes in GABA levels. Such an effect on brain white matter was previously reported (Qiao et al., 2000).

Long-term exposure to VGB at a similar dose in slightly older rats (P12–P26) by Qiao et al. (2000), resulted in short-term white matter damage; in addition, short and long-term behavioral hyperactivity was observed. Considering these findings and our present results, which show hypo-activity in mice exposed during P1–7 and P4–14, delicate age sensitivity may be suggested. Moreover, in our present report, hypo-activity was not measured in 7-week-old mice, but only in adult mice (Fig. 4). Another effect of VGB treatment, which was not age sensitive, was the body weight. Reduced body weight was observed in both

treatment groups and was also reported by Qiao et al. (2000). A clue to a possible mechanism is given by Pinilla et al. (2001) showing the involvement of GABA receptors in the regulation of growth hormone secretion. However, it is possible that VGB has an additional mechanism of action, as may be suggested by the delay in fur growth and eyelid opening.

Blockage of GABA-transaminase elevates GABA levels in the brain (Qume et al., 1995; Sheikh and Martin, 1998; Errante and Petroff, 2003). Prenatal exposure to the $GABA_A$ agonist Diazepam induced both behavioral modifications and sex specific effects on the levels of the neurotrophic factor BDNF (Kellogg et al., 2000), which persists throughout the newborn's life span. This long-lasting effect of early, short GABA enhancement may involve the long-lasting modulation of other factors critical for accurate brain development. The activation of $GABA_A$ receptor by a different $GABA_A$ agonist, Muscimol, at P0 and P1, increased cell death in different regions of the hippocampus at P7 (Nunez et al., 2003a). At P21, a similar treatment reduced the number of cells in the hippocampus. In addition, at this age a minor influence on the performance of mice in the Morris water maze test was observed (Nunez et al., 2003b). These findings may suggest that the effects of VGB treatment on mouse behavior observed in our present study may be at least partially mediated by the enhanced stimulation of the $GABA_A$ receptor. However, contrary to the previous report (Nunez et al., 2003b), we found only transient morphological modifications in the hippocampus (Fig. 6), while in both P21 and adult brains, CA1 and DG cell-layer width was similar in the VGB and control groups. Enhanced apoptotic cell death in various brain regions was observed shortly after treatment with various AEDs, including Diazepam and VGB, as reported by Bittigau et al. (2002). In accordance with this report, low cell density in the cerebral cortex of the VGB4–14 group of adult mice (Fig. 7) may be a result of facilitated cell death induced by the early VGB treatment.

Finally, the apparent impairment of object recognition, spatial learning and memory, demonstrated in the present study suggests damage to the hippocampus (Morris et al., 1982). This was not found by the histological study, indicating their possible regulation at a different level; potential candidates such as synapses, inhibitory efficacy or neurotrophic factors may be considered.

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