

Prenatal Exposure to Carbamazepine Reduces Hippocampal and Cortical Neuronal Cell Population in New-Born and Young Mice without Detectable Effects on Learning And Memory

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Abstract

Pregnant women with epilepsy have to balance maternal and fetal risks associated with uncontrolled seizures against the potential teratogenic effects from antiepileptic drugs (AEDs). Carbamazepine (CBZ) is among the four most commonly used AEDs for treatment of pregnant epileptic women. We previously reported that new-born children had a decreased head circumference after *in utero* CBZ exposure. This study investigates how prenatal exposure of CBZ influences the number of neurons in new-born and young mouse hippocampus, amygdala and cortex cerebri. Clinical studies describe inconclusive results on if prenatal CBZ treatment influences cognition. Here we investigate this issue in mice using two well characterized cognitive tasks, the passive avoidance test and the Morris water maze test. Prenatal exposure of CBZ reduced the number of neurons (NeuN-immunoreactive cells) in the new-born mouse hippocampus with 50% compared to non-exposed mice. A reduction of neurons (20%) in hippocampus was still observed when the animals were 5 weeks old. These mice also displayed a 25% reduction of neurons in cortex cerebri. Prenatal CBZ treatment did not significantly impair learning and memory measured in the passive avoidance test and in the Morris water maze. However, these mice displayed a higher degree of thigmotaxic behaviour than the control mice. The body weight of prenatally CBZ exposed five-week old mice were lower compared to control mice not exposed to CBZ ($p=0.001$). In conclusion, prenatal exposure to CBZ reduces the number of neurons dramatically in areas important for cognition such as hippocampus and cortex, without severe impairments on learning and memory. These results are in line with some clinical studies, reporting that CBZ has minor negative effects on cognition. The challenge for future studies are to segment out what possible effects a reduction of neurons could have on different types of cognition, like intellectual ability and social interaction.

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Introduction

Epilepsy affects 1–2% of humans worldwide and one third of those are women in reproductive age [1,2]. Both seizures and antiepileptic drug (AED) treatment during pregnancy are thought to influence the child negatively. The risk of major fetal congenital malformations as a cause of the disease is approximately 1–2% [3,4] but this risk is even 2–3 fold higher in women with epilepsy treated with AEDs [5,6,7,8]. Thus, pregnant women with epilepsy have to balance maternal and fetal risks associated with uncontrolled seizures against the potential teratogenic effects from AEDs. The most commonly used AEDs for treatment of pregnant epileptic women today are valproate (VPA), phenytoin (PHT), carbamazepine (CBZ) and lamotrigine (LTG) [9].

AEDs are not only subscribed to individuals with epilepsy, but are also used as mood stabilizers in psychiatric disorders [10]. In addition, pregnancy increases the risk for the onset and recurrence

of several psychiatric disorders and pregnant women suffering from affective disorders do often require treatment with mood stabilizers (lithium carbonate, VPA, CBZ, LTG), antidepressants and/or antipsychotics [11,12].

Congenital heart disease, cleft lip and/or palate, limb defects, genitourinary malformations and neural tube defects are malformations seen after AED treatment [13,14,15,16]. Also cognitive impairments, intellectual disability and maladaptive behaviors are associated with exposure to AEDs *in utero* [9,17,18,19,20,21]. It is widely accepted that VPA is associated with more side effects compared to the other commonly used AEDs and that polytherapy regimes are more harmful than monotherapy regimes [9,17,18,19,21,22,23,24,25,26,27,28,29]. Long-term effects into adulthood induced by *in utero* exposure of AEDs have not been extensively studied, even though there are some follow up studies indicating that almost all AEDs have negative effects on later

intellectual functioning [20,30,31,32,33]. Moreover, the mechanisms behind cognitive impairments caused by AEDs are still not well described although a few animal studies have given some clues [34,35,36].

We made a retrospective study on all Swedish singletons births between 1995 and 2005 (over 900,000 births) obtained from the Swedish medical birth registry, and demonstrated a reduced head circumference at birth in newborns exposed to CBZ *in utero* [37]. Previous studies of cognitive performances in young children (up till 6 years of age) exposed to CBZ *in utero* demonstrate conflicting results. Some investigations demonstrate general developmental delay, impaired language and IQ scores [38,39,40,41], while other studies could not detect any cognitive difference in performances between CBZ exposed children and controls [20,29,42,43].

The present study was conducted to investigate whether *in utero* exposure to CBZ can affect the neuronal hippocampal cell population in newborn mice. In addition, we examined the effect of prenatal CBZ exposure on neuronal cell populations in hippocampus, cortex cerebri and amygdala in five-week old mice and related these changes to cognitive performances. Cognitive performance was evaluated by use of the Passive Avoidance (PA) test and the Morris water maze test, which are well characterised cognitive tasks of emotional [44,45] respective spatial learning and memory. Two different mouse strains (BALB/c mice and C57BL/6 mice) were used in this study. The BALB/c mice were used in the PA test and for histological analysis, but since they were poorly performers in the Morris water maze test, we used C57BL/6 mice for this task.

Materials and Methods

Ethics Statement

All animals were handled according to guidelines approved by a local ethics committee (Stockholm Northern Ethics Board of Animal Experimentation) in Sweden.

Animals

Male and female mice, BALB/c and C57BL/6 (Scanbur, Sweden; Taconics, Germany) were used to produce offspring examined in this study. They were housed, 2–7/cage (offspring) or 2–3/cage (pregnant dams), in standard transparent M3 Makrolon® cages lined with bedding material (Scanbur's Aspen wood Bedding, Scanbur, Sweden) in a light/temperature/humidity-controlled environment: 12-h light–dark cycle (light on at 06:00 h), temperature 22°C and 40–50% humidity. Tap water and food pellet (R34 pellet or CBZ diet, Lantmännen, Sweden) was provided ad libitum.

Exposure to CBZ. Starting one week before a male mouse was placed in the cage, the female mice were given CBZ diet (3.5 g CBZ/kg in normal R34 pellet, Lantmännen, Sweden) up to the time of delivery. CBZ diet was replaced with normal R34 pellets, without CBZ, directly after delivery. This CBZ diet has been shown to result in serum levels of CBZ in mice well above the minimal effective dose in humans, but below the optimal therapeutic range for human antiepileptic therapy [46]. The control female mice received normal R34 pellet, (Lantmännen, Sweden) during the same time period. The body weight of the newborn pups was not recorded to avoid disturbing the dam and her pups. For the same reasons the pregnant dams were not monitored for signs of toxicity. However, the numbers of litters and the number of survived pups were noted when the mice were three weeks old. The body weight was measured at five weeks of age.

Female and male BALB/c offspring were used to investigate the effect of prenatal CBZ exposure on the hippocampal and cortical neuronal cell counts in newborn (n = 15) and in young (five weeks old, n = 15) mice. Another group of 5 weeks old male and female BALB/c mice was used to investigate the effect of prenatal CBZ exposure on the cognitive abilities in the PA test (n = 19). Two separate groups of female and male C57BL/6 offspring were used to investigate the effect of prenatal CBZ exposure on spatial learning and memory in the Morris water maze test (age: five weeks (n = 11) and nine weeks (n = 10)). The C57BL/6 mice were used, since BALB/c mice were poor performers in this test.

Perfusion

Newborn pups were decapitated and the brains dissected and incubated in fixative solution (4% paraformaldehyde and 0.4% picric acid in 0.16 M PBS, pH 7.4) for 2 h at room temperature (RT). The brains were then rinsed and stored in sucrose (10% in PBS, 0.1% sodium azide) at 4°C and thereafter snap-frozen in dry-ice-cooled iso-pentane.

5 weeks old mice were deeply anesthetized by pentobarbital and then perfused intracardially at forced pressure with 10 ml Ca²⁺-free Tyrode's solution including 0.1 ml heparin, followed by 50 ml of the fixative solution. The brains were dissected and postfixed in the same fixative for 1 h at RT, rinsed and stored in the sucrose solution at 4°C, and thereafter snap-frozen.

Immunohistochemistry

The brains were cryosectioned in 30 µm coronal sections encompassing the entire hippocampus in a cryostat and mounted on Super-Frost slides. Mouse on mouse (M.O.M) kit for immunodetection (Vector, Burlingame, CA) was used for NeuN staining according to the manufacturer's protocol. The sections were incubated with the primary antibody (anti-NeuN, 1:100, Chemicon) for 24 h at 4°C and with biotinylated secondary antibody (anti-mouse IgG, 1:250, M.O.M kit) for 1 h in RT. To enhance signals, ABC Vectastain Elite Reagent (Vector) was applied for 40 min at RT. After detection with 3'3-diaminobenzidine substrate (DAB, Sigma) for 30 seconds in RT the slides were dehydrated and mounted with Pertex (Histolab, Gothenburg Sweden).

Stereology

The "Optical fractionator" [47,48] was used to estimate the number of neurons in the dentate gyrus (DG), in the CA1 and CA3 regions of the hippocampus, in cortex cerebri and in amygdala of CBZ exposed and control mice. In newborn mice, neuronal cell counts were determined in the rostral part of hippocampus (−3.5 mm from Bregma to −4.5 mm from Bregma) [49] combining the hippocampal regions CA1 and CA3. Cell counts in five weeks old mice were determined through the entire hippocampus (−0.9 mm from Bregma to −3.9 mm from Bregma) [50]. Here, neurons in DG were counted as one entity whereas neuron counts from the CA1 and CA3 regions were combined. Neuronal cell counts in cortex cerebri and amygdala (−1.6 mm from Bregma to −2.1 mm from Bregma) were also counted [50].

Every tenth section was systematically sampled after that the first section within the first interval had been chosen randomly. An unbiased counting frame of known area was superimposed on the field of view by stereological analysis software (Stereologer™, SPA inc., VA, USA). These counting frames were then automatically and systematically distributed throughout the marked region originating from a random starting point. The optical fractionator estimates are unbiased, since no assumptions are made about cellular shape and size, and not affected by tissue shrinkage during

tissue processing. Before counting an area of interest, the areas were manually outlined using a 10× lens; whereas a 60× lens with a numerical aperture of 1.4 was used for cell counts. Only cells that fell within the frame or bordered the inclusion lines were counted, while cells touching exclusion lines were omitted. These steps were performed with a light microscope (Olympus BH-2) modified for stereology with the following components: a computer-driven motorized Märzhäuser stage (Märzhäuser Wetzlar GmbH & Co. KG, Wetzlar-Steindorf, Germany), a microcator (Heidenhain, Traunreut, Germany) and a CCD camera. All stereological analyses were conducted in one hemisphere of the brain.

Passive Avoidance

The PA task is an associative learning paradigm based on contextual fear conditioning (Pavlovian conditioning), involving neuronal circuits in the limbic forebrain, such as hippocampus and amygdala [44,51]. In the step-through PA procedure, performed in a two-compartment box, the suppression of the innate preference of rodents for the dark compartment following the exposure to an inescapable foot shock is defined as PA behavior [44,45,52,53]. Memory retention was tested in a computer-controlled PA (TSE-Systems GmbH, Homburg, Germany).

The experiments were performed between 8 a.m. and 3 p.m. Test of memory retention was performed 24 hours after the training [52,53]. During training the mouse was placed in a bright compartment (BC, 330 lux) (280×155×160 mm) for 60 s. Then the door between the compartments opens and the mouse has free access to the dark compartment (DC) (280×155×160 mm). Upon entering the DC the door closes after 3 s and the mouse received weak electrical current (US) (duration 1 s) 0.30 mA. The mouse was left in the dark compartment for 60 s after the aversive cue (US) had been presented to increase the association of the context and the US [52,53]. In the retention test the mouse was placed in the BC with the door closed. After 15 s the door was opened and the mouse had free access to both compartments for 10 min, 600 s. Memory retention was examined by measuring the latency time to the first transfer from the BC to the DC with a cut-off latency of 10 min (600 s) [45,52,53]. After the end of each test, the arena was cleaned and deodorised after each animal using 70% ethanol.

Morris Water Maze

The water maze (180 cm in diameter; 45 cm in height) was filled to a depth of 28 cm with tap water (23°C) and placed in the center of a room surrounded by several cues. All studies were performed under dim light conditions (Ögren et al., 1996). Pre-training was performed during three days followed by training during six days, both with four 90 s long trials/day. A 60 s long retention test was performed twenty-four hours after the last training session.

During pre-training the platform (10 cm in diameter) was located 1 cm below the water surface, but was made visible for the animals. The platform location was changed every trial and the periphery of the water tank was covered by a curtain to prevent access to extra-maze cues. During training the platform was located 1 cm below the water surface and invisible to the animal. The platform location remained constant over the six days of training, but was removed at retention on day seven. Before starting the experiments the mice were allowed to habituate to the water maze room for one hour. The water maze system (Water Maze Software, Edinburgh, UK) allow measurements of latency to find the platform, swim distance, swim speed, thigmotaxic behavior as well as quadrant and zone analyses (Cain et al.,

1996; Steele and Morris, 1999; Luttgen et al., 2005). A circular area (radius 20 cm from the center of the platform) was defined as the target zone, equivalent to 4.9% of the total water maze area. Thigmotaxic swimming, was defined as the percentage of total time the mice spent swimming within 10 cm from the walls of the water maze.

Statistical analysis

The results are presented as means ±SEM. The data were analyzed for normality. Normally distributed data were analyzed with parametric tests. Differences between groups were tested with one way-analysis of variance (ANOVA) in the PA task. Student's *t* test was applied to analyse cell counting data from both new-born and five-week old animals.

The behavioural data were first visually assessed for normality by the sample distribution. Normally distributed results were analysed with parametric test. Differences between the groups were tested with one way- analysis of variance (ANOVA). Significant differences between groups were tested with the Fisher LSD test. The nonparametric test Kruskal Wallis was used for data that were not normally distributed. $P < 0.05$ was regarded as statistically significant. All data were analyzed with the computer software program Statistica® 7.0.

Results

Prenatal exposure of carbamazepine reduced the number of hippocampal pyramidal neurons in newborn BALB/c mice with 50%

Prenatal CBZ exposure reduced the number of mature neurons (NeuN-immunoreactive cells) in the CA1 and CA3 regions of hippocampus ($P = 0.0013$, Fig. 1A) by 50% compared to the control group not exposed to CBZ. There were no mature neurons in the dentate gyrus in the newborn mice (Fig. 1B).

Prenatal CBZ exposure had long-term effects on hippocampal and cortical pyramidal cells in young BALB/c mice

Prenatal CBZ exposure reduced the number of mature neurons in hippocampal CA1 and CA3 regions in 5 week-old mice, compared to non-exposed mice ($P = 0.0004$, Fig. 2A). CBZ exposure had no effect on neurons in the dentate gyrus (data not shown).

Stereological counting of NeuN-positive neurons in cortex cerebri revealed that the number of mature neurons was lower in mice that had been exposed to CBZ *in utero* than in non-exposed mice, ($P = 0.0008$, Fig. 3A). However, CBZ did not affect neurons in amygdala.

Prenatal CBZ exposure had no effect on learning and memory in young mice

The step-through latency retention in the passive avoidance test was similar between CBZ-exposed and non-exposed BALB/c mice demonstrating that prenatal treatment with CBZ had no effect on amygdala-hippocampal-dependent emotional type of learning and memory (Fig. 4). Likewise, the results from the Morris water maze test showed that prenatal CBZ exposure had no effect on hippocampal-dependent spatial learning and memory (Fig. 5). However, the CBZ exposed C57BL/6 mice displayed an anxious and thigmotaxic behaviour demonstrated by that they spent more time closely to the walls of the pool compared to the non-exposed mice ($P = 0.0092$, Fig. 5D, H).

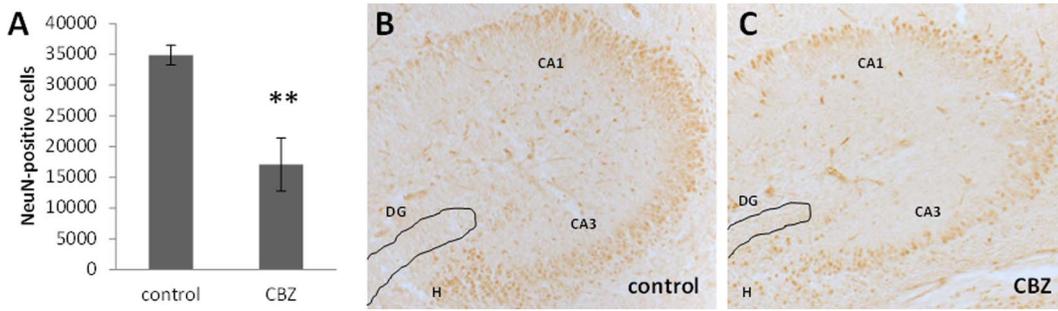


Figure 1. The effect from *in utero* exposure to CBZ on newborn mice. A) Prenatal exposure of CBZ reduces the number of mature pyramidal neurons in hippocampal CA1 and CA3 regions in the newborn mouse. A newborn control mouse hippocampus (B) and a CBZ treated mouse (C). The brown cells (NeuN-immunoreactive cells), which are indicative of mature neurons are more frequently occurring in the non-treated animals (B). There are few NeuN-immunoreactive cells in the newborn mouse dentate gyrus (DG), in agreement with that granule cells mature at later developmental stages compared to pyramidal cells in the CA1 and CA3 regions and in the hilus (H) region. The analysis is performed in the dorsal hippocampus. Values are mean \pm SEM. n=8 controls, n=7 CBZ treated animals, ** $P=0.0013$. Scale bar: B) 100 μ m C) 25 μ m. doi:10.1371/journal.pone.0080497.g001

Prenatal treatment with CBZ decreased the number of litters, weaned pups and body weight

The number of litters/dam was reduced two times as a result of CBZ exposure during pregnancy. Non-exposed dams had 4 litters/dam during the breeding period while the CBZ exposed dams only had 2 litters/dam. The average number of weaned pups/dam and litter was lower in the group prenatally exposed to CBZ (2.8 pups/dam and litter) compared to non-exposed dams (7.5 pups/dam and litter). In addition, five weeks old mice prenatally exposed to CBZ had a significantly lower body weight than non-exposed mice ($P=0.001$, Table 1).

Discussion

This is the first study, to our knowledge, investigating the effects of prenatal exposure of CBZ on hippocampal, amygdala and cortical neurons, which are indeed important areas for learning and memory. The major findings in this study are that prenatal exposure of CBZ reduces the number of neurons in the new-born mouse hippocampus, indicated by a 50% reduction of NeuN-immunoreactive cells and that prenatal exposure to CBZ also affects the cell population in five-week old mouse hippocampus

and cortex cerebri. Thus, the neurodegenerative effects from prenatal CBZ exposure is long-lasting and are not restricted to hippocampus but also found in cortex cerebri. Interestingly, reduction of neurons were found in hippocampal CA1 and CA3 and cortex cerebri pyramidal cells but not in granule cells in the dentate gyrus and medium spiny neurons in the amygdala. Some cell types may be more sensitive to teratogens than others, in agreement with for example Charriaut-Marlangue et al., Crepel et al and Pellegrini-Giampietro et al. [54,55,56]. Since granule cells primarily develop during the early postnatal period, which is after CBZ exposure, their development may have been unaffected [57,58]. In addition, Cochrane (2009) suggests that AED exposure *in utero* can achieve a precise type of cognitive disturbance by generating a more specific or strong effect in certain brain areas compared to others [9].

Based on the inconclusive results in clinical studies of the effect of prenatal CBZ exposure on cognition [17,20,21,29,38,39,40,41,42,43] we examined the effect of prenatal CBZ exposure on learning and memory in young (5 week-old) BALB/c mice using the Passive Avoidance (PA) test which involves neuronal circuits in the limbic forebrain, such as hippocampus and amygdala. Our intention was, in addition to the PA test, to also

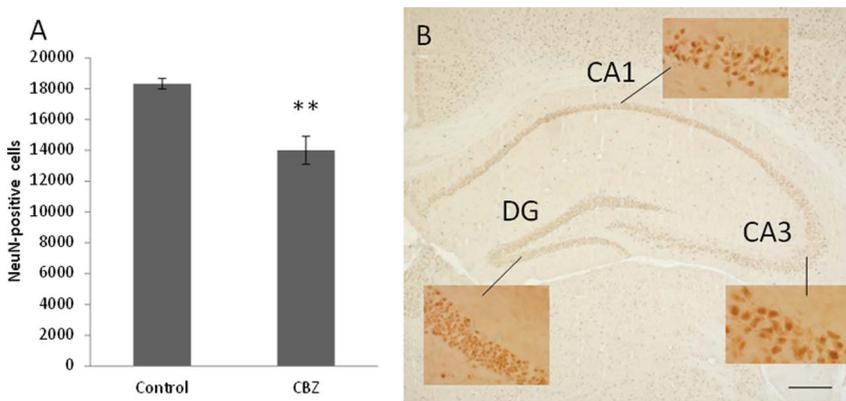


Figure 2. The effect from *in utero* exposure to CBZ on hippocampus in 5 week-old mice. A) Down-regulation of hippocampal CA1 and CA3 pyramidal neurons after prenatal exposure of CBZ are still found in 5 week-old mice. Prenatal CBZ exposure does not affect neurons (granule cells) in the dentate gyrus (DG). B) A representative picture of hippocampus from one brain hemisphere in a five week-old mouse (scale bar 250 μ m). High-magnification images illustrate cell morphology of granule cells in DG and pyramidal cells in CA1 and CA3, where the stereological cell-counting were performed. n=8 controls, n=7 CBZ treated animals, ** $P=0.0004$. doi:10.1371/journal.pone.0080497.g002

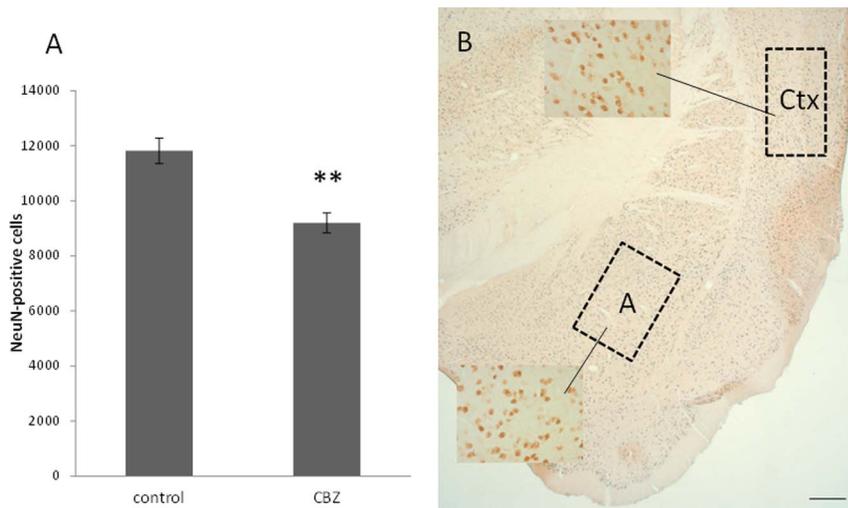


Figure 3. The effect from *in utero* exposure to CBZ is detected in different parts of the brain. A) Prenatal exposure of CBZ decreases the number of pyramidal cells in cortex cerebri of five-week old mice. CBZ did not alter neuronal number in amygdala. B) Representative picture of one brain hemisphere in a five-week old mouse at the level where stereological counting was performed in cortex cerebri (Ctx) and amygdala (A). High-magnification images illustrate cell morphology of pyramidal cells in cortex cerebri and spiny neurons in amygdala (scale bar 250 μ m). n = 8 controls, n = 7 CBZ treated animals, ** $P = 0.0008$ doi:10.1371/journal.pone.0080497.g003

investigate BALB/c mice in the Morris water maze task, which is mainly a hippocampus-dependent spatial learning and memory task [59,60]. However, the BALB/c mice displayed an abnormally high amount of thigmotactic behaviour, in which they swam around the walls of the pool almost continuously, without any attempt to reach the platform and to get out of the pool. The behaviour could be viewed as a displacement behaviour, displayed in place of other behaviours, when neither being in the water nor out in the open was enough rewarding to perform. The desire to avoid the open of the pool mirrors a higher level of emotionality in this mouse strain. The BALB/c is considered to be a more anxious mouse strain than the C57BL/6 [61,62]. Since the escape latency in BALB/c could be due to both anxiety and cognition we evaluated the effect of prenatal CBZ exposure on spatial cognition

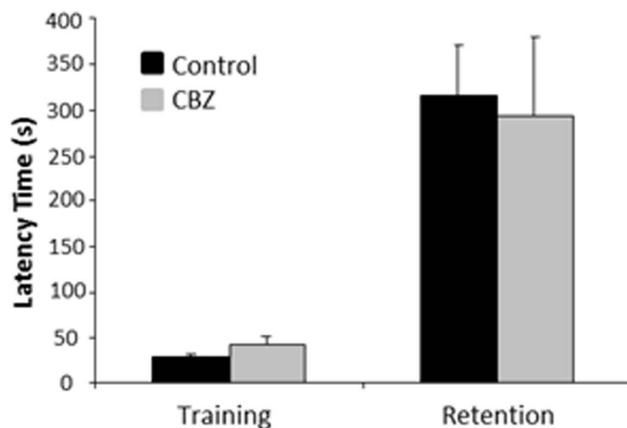


Figure 4. *In utero* exposure to CBZ did not alter emotional memory in 5 week-old mice investigated in the passive avoidance test. There was no effect on training latency or retention latency by prenatal CBZ-exposure. The black bars indicate non-exposed mice (n = 13) whereas the grey bars indicate CBZ-exposed mice (n = 6). Bars indicate mean values and error bars indicate \pm SEM. doi:10.1371/journal.pone.0080497.g004

using the C57BL/6 in the Morris water maze. Prenatal CBZ treatment had no detectable effects on learning and memory, neither in the PA nor in the Morris water maze test. These results are in line with most of the clinical investigations performed, which demonstrate that CBZ has minor effects on cognition, especially compared to VPA [17,20,42,43].

Prenatally CBZ treated C57BL/6 mice also displayed the anxiety-like behaviour, thigmotaxis, in the Morris water maze. Whether this is due to a direct CBZ toxic effect on the neural circuits involved in regulating anxiety or an overall effect of CBZ on homeostasis is unknown. It could also be due to fewer cage mates (or occasionally none) for the CBZ exposed animals. The decreased body weight found in the prenatally treated mice could be associated with the increased anxiety level in these animals. Decreased body weight and increased level of anxiety could also separately be a result of CBZ toxicity mentioned in the below section. Whether CBZ has a general toxic effect on the brain neuronal network, or if it hits specific networks, is a question for further investigations.

Pregnant dams treated with CBZ got two times fewer litters/dam compared to non-exposed dams. The average number of weaned pups/dam and litter was also lower in the group prenatally exposed to CBZ compared to non-exposed dams recorded at an age of 3 weeks. In addition, prenatal CBZ exposure also decreased the body weight recorded at five-weeks of age. The decreased number of weaned pups prenatally exposed to CBZ could be due to impaired mating, impaired survival of embryos, absorption of foetuses, less nutrition during the gestation period, complicated parturition, impaired maternal care as well as impaired feeding behaviour or success in competition for food. The role of CBZ in different stages of development as well as on the maternal behaviour and pup response needs to be further elucidated.

CBZ has been demonstrated to induce mitotic arrest in human kidney-derived cell lines [63]. Postnatal CBZ administration to rats on day 7/day 8 did not result in detectable effects on cell proliferation, neurogenesis or cell death [35,36]. The effects of CBZ exposure may be different depending on if the exposure is

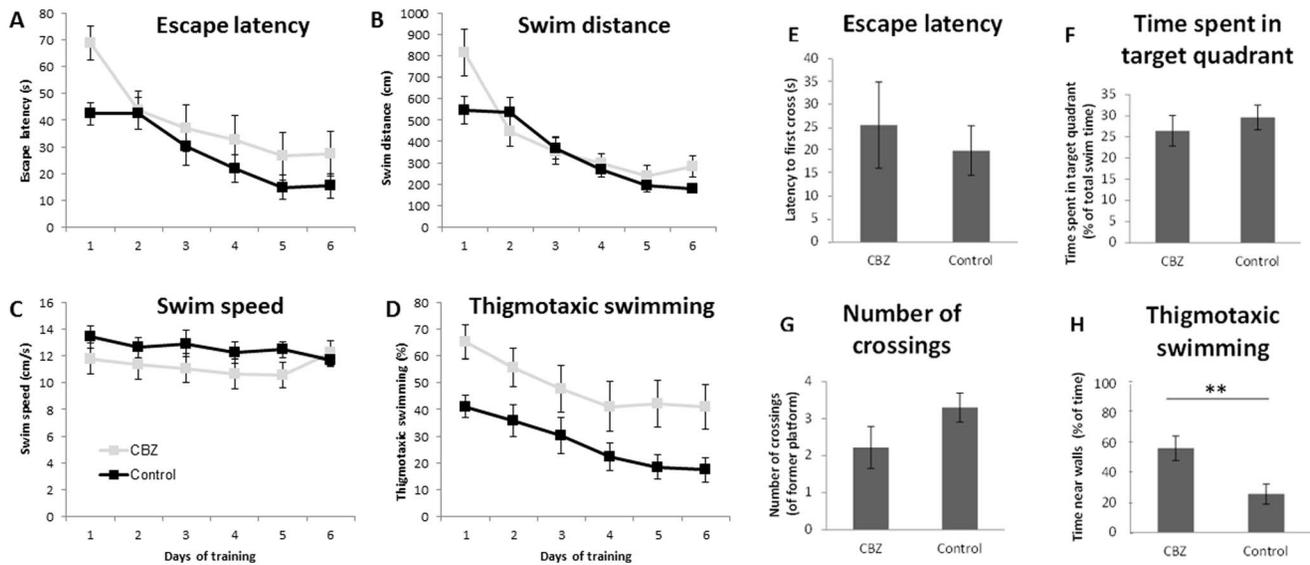


Figure 5. The effects of prenatal CBZ exposure on learning and memory investigated in the Morris water maze test. Figure panels 5 A–D describe the six training days and panels 5 E–H the retention test. *Training sessions.* 5A) There was no significant difference, but a trend, that CBZ-exposed mice were impaired in their ability to improve their escape latency to the hidden platform by training ($P=0.098$). B) The CBZ-exposed mice had a significantly different swimming distance pattern over training compared to the control mice ($P=0.025$). After training number 2, the CBZ-exposed mice reduced their swimming distance less by training than the control mice did. C) There was no significant difference in change of swimming speed over time between the CBZ-exposed mice and the controls during training ($P=0.39$). D) Although there was no effect of training on the time the mice in both groups swam near the walls of the pool (thigmotaxic swimming) ($P=0.44$), the CBZ-exposed mice tended to have higher thigmotaxic swimming all the days of training ($P=0.073$). *Retention.* 5E) There was no significant difference in time for the CBZ-exposed and the control mice to find the former platform position, escape latency ($P=0.44$). F) The two groups of mice spent the same proportion of total swim time in the target quadrant, where the former platform position was ($P=0.38$). G) The number of times crossing the platform position did not differ between CBZ-exposed and control mice ($P=0.79$). H) Thigmotaxic swimming; CBZ-exposed mice swam significantly longer time near the walls of the pool during retention ($P=0.0092$) compared to controls, suggesting that they were more stressed. Bars indicate mean values and error bars indicate \pm SEM. ** $P<0.01$, $n=10$ CBZ-exposed mice, $n=11$ control mice. doi:10.1371/journal.pone.0080497.g005

postnatal or prenatal. The reduction of neurons found in this investigation could possibly be caused by a decrease in cell proliferation, altered neurogenesis or neurodegeneration. AEDs can down-regulate neurotrophic factors, such as BDNF and NT-3 [34]. It is thus possible that CBZ down-regulated the trophic support for neurons and that this in turn caused neuronal death. AEDs have also been demonstrated to alter intracellular pathways involved in survival promoting signalling. Further studies are needed to address possible mechanisms behind the decrease of the neuronal cell population found after prenatal CBZ exposure.

This is the first study to investigate prenatal CBZ treatment and the effects on brain neuronal cell populations in combination with learning and memory. However, there are some limitations in this study. 1. As already mentioned, BALB/c could be used only in one of the learning and memory tests performed. However, similar to the lack of detectable effect of prenatal CBZ exposure on

emotional learning in BALB/c, there was no effect on spatial learning in the C57BL/6 mice. 2. Toxicity of CBZ in pregnant mice or CBZ effects on new-borns, were not investigated in this study. Body weight was only recorded in five-week old mice. The CBZ dose used has been shown to result in serum levels of CBZ well above the minimal effective dose in humans, but below the optimal therapeutic range for human antiepileptic therapy [46]. 3. The CBZ-exposed mice were fewer than those not exposed to CBZ in the passive-avoidance test. Still, adequate statistical models and analyses were performed.

The last Cochrane review (2009) stated the necessity to generate data establishing the effects of *in utero* AED exposure on child development and cognition [9]. When translating the findings of our study to humans the differences in brain developmental stages between rodents and humans should be considered, where the human brain goes through more stages *in utero* than the rodent brain does [64,65] and also the longer drug-exposure time *in utero* for human fetuses compared to rodent fetuses.

In conclusion, the present study demonstrates that *in utero* exposure to CBZ produces a drastic reduction of mature hippocampal and cortical neurons. Interestingly, no apparent cognitive impairment was detected in the PA test or in the Morris water maze despite this huge reduction of neuron count. This proposes that the loss of neurons in brain areas important for cognition possibly can be compensated for by long-term structural rearrangements of neuronal networks. However, another interpretation may be that CBZ does not affect emotional memory and spatial learning, but rather influence other aspects of cognition like intellectual ability and social interaction. Further studies are

Table 1. Prenatal exposure to carbamazepine (CBZ) influences mouse body weight. Exposed mice had a lower body weight than non-exposed mice.

	BALB/c [5 weeks]
CBZ-exposed mice	14.7 \pm 0.48 (6)***
Non-exposed mice	17.7 \pm 0.30 (13)

The weight is presented in grams and as mean \pm SEM (n).
 *** $P<0.001$ compared to non-exposed mice.
 doi:10.1371/journal.pone.0080497.t001

required to scrutinize the influence of the CBZ-caused loss of neurons on cognitive functions.

References

- Hauser WA (1994) The prevalence and incidence of convulsive disorders in children. *Epilepsia* 35 Suppl 2: S1–6.
- Wallace H, Shorvon S, Tallis R (1998) Age-specific incidence and prevalence rates of treated epilepsy in an unselected population of 2,052,922 and age-specific fertility rates of women with epilepsy. *Lancet* 352: 1970–1973.
- Crawford P, Appleton R, Betts T, Duncan J, Guthrie E, et al. (1999) Best practice guidelines for the management of women with epilepsy. The Women with Epilepsy Guidelines Development Group. *Seizure: the journal of the British Epilepsy Association* 8: 201–217.
- Olafsson E, Hallgrímsson JT, Hauser WA, Ludvigsson P, Gudmundsson G (1998) Pregnancies of women with epilepsy: a population-based study in Iceland. *Epilepsia* 39: 887–892.
- Holmes LB, Harvey EA, Coull BA, Huntington KB, Khoshbin S, et al. (2001) The teratogenicity of anticonvulsant drugs. *The New England journal of medicine* 344: 1132–1138.
- Samren EB, van Duijn CM, Koch S, Hillesmaa VK, Klepel H, et al. (1997) Maternal use of antiepileptic drugs and the risk of major congenital malformations: a joint European prospective study of human teratogenesis associated with maternal epilepsy. *Epilepsia* 38: 981–990.
- Samren EB, van Duijn CM, Christiaens GC, Hofman A, Lindhout D (1999) Antiepileptic drug regimens and major congenital abnormalities in the offspring. *Annals of neurology* 46: 739–746.
- Kaneko S, Battino D, Andermann E, Wada K, Kan R, et al. (1999) Congenital malformations due to antiepileptic drugs. *Epilepsy research* 33: 145–158.
- Adab N, Tudur SC, Vinten J, Williamson P, Winterbottom J (2004) Common antiepileptic drugs in pregnancy in women with epilepsy. *Cochrane database of systematic reviews*: CD004848.
- Galbally M, Roberts M, Buist A (2010) Mood stabilizers in pregnancy: a systematic review. *The Australian and New Zealand journal of psychiatry* 44: 967–977.
- Gentile S (2011) Drug treatment for mood disorders in pregnancy. *Current opinion in psychiatry* 24: 34–40.
- Gentile S (2010) Neurodevelopmental effects of prenatal exposure to psychotropic medications. *Depression and anxiety* 27: 675–686.
- Kaplan PW, Norwitz ER, Ben-Menachem E, Pennell PB, Druzin M, et al. (2007) Obstetric risks for women with epilepsy during pregnancy. *Epilepsy & behavior: E&B* 11: 283–291.
- Meador KJ, Pennell PB, Harden CL, Gordon JC, Tomson T, et al. (2008) Pregnancy registries in epilepsy: a consensus statement on health outcomes. *Neurology* 71: 1109–1117.
- Pennell PB (2004) Pregnancy in women who have epilepsy. *Neurologic clinics* 22: 799–820.
- Morrow JI, Hunt SJ, Russell AJ, Smithson WH, Parsons L, et al. (2009) Folic acid use and major congenital malformations in offspring of women with epilepsy: a prospective study from the UK Epilepsy and Pregnancy Register. *Journal of neurology, neurosurgery, and psychiatry* 80: 506–511.
- Meador KJ, Baker G, Cohen MJ, Gaily E, Westerveld M (2007) Cognitive/behavioral teratogenic effects of antiepileptic drugs. *Epilepsy & behavior: E&B* 11: 292–302.
- Meador KJ, Baker GA, Browning N, Clayton-Smith J, Combs-Cantrell DT, et al. (2009) Cognitive function at 3 years of age after fetal exposure to antiepileptic drugs. *The New England journal of medicine* 360: 1597–1605.
- Bromley RL, Baker GA, Meador KJ (2009) Cognitive abilities and behaviour of children exposed to antiepileptic drugs in utero. *Current opinion in neurology* 22: 162–166.
- Adab N, Kini U, Vinten J, Ayres J, Baker G, et al. (2004) The longer term outcome of children born to mothers with epilepsy. *Journal of neurology, neurosurgery, and psychiatry* 75: 1575–1583.
- Pennell PB (2008) Antiepileptic drugs during pregnancy: what is known and which AEDs seem to be safest? *Epilepsia* 49 Suppl 9: 43–55.
- Harden CL, Meador KJ, Pennell PB, Hauser WA, Gronseth GS, et al. (2009) Management issues for women with epilepsy-Focus on pregnancy (an evidence-based review): II. Teratogenesis and perinatal outcomes: Report of the Quality Standards Subcommittee and Therapeutics and Technology Subcommittee of the American Academy of Neurology and the American Epilepsy Society. *Epilepsia* 50: 1237–1246.
- Meador KJ, Penovich P, Baker GA, Pennell PB, Bromfield E, et al. (2009) Antiepileptic drug use in women of childbearing age. *Epilepsy & behavior: E&B* 15: 339–343.
- Banach R, Boskovic R, Einarson T, Koren G (2010) Long-term developmental outcome of children of women with epilepsy, unexposed or exposed prenatally to antiepileptic drugs: a meta-analysis of cohort studies. *Drug safety: an international journal of medical toxicology and drug experience* 33: 73–79.
- Vinten J, Bromley RL, Taylor J, Adab N, Kini U, et al. (2009) The behavioral consequences of exposure to antiepileptic drugs in utero. *Epilepsy & behavior: E&B* 14: 197–201.
- Vinten J, Adab N, Kini U, Gorry J, Gregg J, et al. (2005) Neuropsychological effects of exposure to anticonvulsant medication in utero. *Neurology* 64: 949–954.
- Eriksson K, Viinikainen K, Monkkonen A, Aikia M, Nieminen P, et al. (2005) Children exposed to valproate in utero—population based evaluation of risks and confounding factors for long-term neurocognitive development. *Epilepsy research* 65: 189–200.
- Thomas SV, Ajaykumar B, Sindhu K, Nair MK, George B, et al. (2008) Motor and mental development of infants exposed to antiepileptic drugs in utero. *Epilepsy & behavior: E&B* 13: 229–236.
- Adab N, Jacoby A, Smith D, Chadwick D (2001) Additional educational needs in children born to mothers with epilepsy. *Journal of neurology, neurosurgery, and psychiatry* 70: 15–21.
- Titze K, Koch S, Helge H, Lehmkühl U, Rauh H, et al. (2008) Prenatal and family risks of children born to mothers with epilepsy: effects on cognitive development. *Developmental medicine and child neurology* 50: 117–122.
- Dessens A, Cohen-Kettenis P, Mellenbergh G, van de Poll N, Koppe J, et al. (1998) Prenatal exposure to anticonvulsant drugs and spatial ability in adulthood. *Acta neurobiologicae experimentalis* 58: 221–225.
- Sulzbacher S, Farwell JR, Temkin N, Lu AS, Hirtz DG (1999) Late cognitive effects of early treatment with phenobarbital. *Clinical pediatrics* 38: 387–394.
- Reinisch JM, Sanders SA, Mortensen EL, Rubin DB (1995) In utero exposure to phenobarbital and intelligence deficits in adult men. *JAMA: the journal of the American Medical Association* 274: 1518–1525.
- Bitigau P, Sifringer M, Genz K, Reith E, Pospischil D, et al. (2002) Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. *Proceedings of the National Academy of Sciences of the United States of America* 99: 15089–15094.
- Chen J, Cai F, Cao J, Zhang X, Li S (2009) Long-term antiepileptic drug administration during early life inhibits hippocampal neurogenesis in the developing brain. *Journal of neuroscience research* 87: 2898–2907.
- Kim J, Kondratyev A, Gale K (2007) Antiepileptic drug-induced neuronal cell death in the immature brain: effects of carbamazepine, topiramate, and levetiracetam as monotherapy versus polytherapy. *The Journal of pharmacology and experimental therapeutics* 323: 165–173.
- Almgren M, Kallen B, Lavebratt C (2009) Population-based study of antiepileptic drug exposure in utero—influence on head circumference in newborns. *Seizure: the journal of the British Epilepsy Association* 18: 672–675.
- Jones KL, Lacro RV, Johnson KA, Adams J (1989) Pattern of malformations in the children of women treated with carbamazepine during pregnancy. *The New England journal of medicine* 320: 1661–1666.
- Ornoy A, Cohen E (1996) Outcome of children born to epileptic mothers treated with carbamazepine during pregnancy. *Archives of disease in childhood* 75: 517–520.
- Dean JC, Hailey H, Moore SJ, Lloyd DJ, Turnpenny PD, et al. (2002) Long term health and neurodevelopment in children exposed to antiepileptic drugs before birth. *Journal of medical genetics* 39: 251–259.
- Cummings C, Stewart M, Stevenson M, Morrow J, Nelson J (2011) Neurodevelopment of children exposed in utero to lamotrigine, sodium valproate and carbamazepine. *Archives of disease in childhood* 96: 643–647.
- Gaily E, Kantola-Sorsa E, Hillesmaa V, Isoaho M, Matila R, et al. (2004) Normal intelligence in children with prenatal exposure to carbamazepine. *Neurology* 62: 28–32.
- Scolnik D, Nulman I, Rovet J, Gladstone D, Czuchta D, et al. (1994) Neurodevelopment of children exposed in utero to phenytoin and carbamazepine monotherapy. *JAMA: the journal of the American Medical Association* 271: 767–770.
- Baarendse PJ, van Groothoest G, Jansen RF, Pieneman AW, Ogren SO, et al. (2008) Differential involvement of the dorsal hippocampus in passive avoidance in C57Bl/6J and DBA/2J mice. *Hippocampus* 18: 11–19.
- Stiedl O, Misane I, Spiess J, Ogren SO (2000) Involvement of the 5-HT_{1A} receptors in classical fear conditioning in C57BL/6J mice. *The Journal of neuroscience: the official journal of the Society for Neuroscience* 20: 8515–8527.
- Lavebratt C, Trifunovski A, Persson AS, Wang FH, Klason T, et al. (2006) Carbamazepine protects against megencephaly and abnormal expression of BDNF and Nogo signaling components in the mceph/mceph mouse. *Neurobiology of disease* 24: 374–383.
- West MJ, Gundersen HJ (1990) Unbiased stereological estimation of the number of neurons in the human hippocampus. *The Journal of comparative neurology* 296: 1–22.

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Conceived and designed the experiments: EÅ SH SOÖ CL. Performed the experiments: EÅ SH. Analyzed the data: EÅ SH AN. Contributed reagents/materials/analysis tools: EÅ SOÖ CL. Wrote the paper: EÅ.

48. West MJ, Slomianka L, Gundersen HJ (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *The Anatomical record* 231: 482–497.
49. Paxinos G, Halliday G, Watson C, Koutcherov Y, Wang H (2007) *Atlas of the Developing mouse brain*. Academic Press, Elsevier.
50. Franklin K, Paxinos G (1997) *The mouse brain in stereotaxic coordinates*. San Diego: Academic Press.
51. LeDoux JE (1993) Emotional memory systems in the brain. *Behavioural brain research* 58: 69–79.
52. Madjid N, Tottie EE, Luttgen M, Meister B, Sandin J, et al. (2006) 5-Hydroxytryptamine 1A receptor blockade facilitates aversive learning in mice: interactions with cholinergic and glutamatergic mechanisms. *The Journal of pharmacology and experimental therapeutics* 316: 581–591.
53. Eriksson TM, Madjid N, Elvander-Tottie E, Stiedl O, Svenningsson P, et al. (2008) Blockade of 5-HT_{1B} receptors facilitates contextual aversive learning in mice by disinhibition of cholinergic and glutamatergic neurotransmission. *Neuropharmacology* 54: 1041–1050.
54. Pellegrini-Giampietro DE, Gorter JA, Bennett MV, Zukin RS (1997) The GluR2 (GluR-B) hypothesis: Ca²⁺-permeable AMPA receptors in neurological disorders. *Trends in neurosciences* 20: 464–470.
55. Charriat-Marlangue C, Aggoun-Zouaoui D, Represa A, Ben-Ari Y (1996) Apoptotic features of selective neuronal death in ischemia, epilepsy and gp 120 toxicity. *Trends in neurosciences* 19: 109–114.
56. Crepel V, Epsztein J, Ben-Ari Y (2003) Ischemia induces short- and long-term remodeling of synaptic activity in the hippocampus. *Journal of cellular and molecular medicine* 7: 401–407.
57. Bayer SA, Altman J (1974) Hippocampal development in the rat: cytogenesis and morphogenesis examined with autoradiography and low-level X-irradiation. *The Journal of comparative neurology* 158: 55–79.
58. Dobbing J, Sands J (1979) Comparative aspects of the brain growth spurt. *Early human development* 3: 79–83.
59. Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *Journal of neuroscience methods* 11: 47–60.
60. D'Hooge R, De Deyn PP (2001) Applications of the Morris water maze in the study of learning and memory. *Brain research Brain research reviews* 36: 60–90.
61. Lepicard EM, Joubert C, Hagneau I, Perez-Diaz F, Chapouthier G (2000) Differences in anxiety-related behavior and response to diazepam in BALB/cByJ and C57BL/6J strains of mice. *Pharmacology, biochemistry, and behavior* 67: 739–748.
62. Ohl F, Sillaber I, Binder E, Keck ME, Holsboer F (2001) Differential analysis of behavior and diazepam-induced alterations in C57BL/6N and BALB/c mice using the modified hole board test. *Journal of psychiatric research* 35: 147–154.
63. Perez Martin JM, Fernandez Freire P, Labrador V, Hazen MJ (2008) Carbamazepine induces mitotic arrest in mammalian Vero cells. *Mutation research* 637: 124–133.
64. Quinn R (2005) Comparing rat's to human's age: how old is my rat in people years? *Nutrition* 21: 775–777.
65. Dwyer JB, McQuown SC, Leslie FM (2009) The dynamic effects of nicotine on the developing brain. *Pharmacology & therapeutics* 122: 125–139.