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FULL-LENGTH ORIGINAL RESEARCH

Long-term outcome in a noninvasive rat model of birth asphyxia with neonatal seizures: Cognitive impairment, anxiety, epilepsy, and structural brain alterations

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Abstract

Objective: Birth asphyxia is a major cause of hypoxic–ischemic encephalopathy (HIE) in neonates and often associated with mortality, neonatal seizures, brain damage, and later life motor, cognitive, and behavioral impairments and epilepsy. Preclinical studies on rodent models are needed to develop more effective therapies for preventing HIE and its consequences. Thus far, the most popular rodent models have used either exposure of intact animals to hypoxia-only, or a combination of hypoxia and carotid occlusion, for the induction of neonatal seizures and adverse outcomes. However, such models lack systemic hypercapnia, which is a fundamental constituent of birth asphyxia with major effects on neuronal excitability. Here, we use a recently developed noninvasive rat model of birth asphyxia with subsequent neonatal seizures to study later life adverse outcome.

Methods: Intermittent asphyxia was induced for 30 min by exposing male and female postnatal day 11 rat pups to three 7 + 3-min cycles of 9% and 5% O_2 at constant 20% CO_2. All pups exhibited convulsive seizures after asphyxia. A set of behavioral tests were performed systematically over 14 months following asphyxia, that is, a large part of the rat's life span. Video-electroencephalographic (EEG) monitoring was used to determine whether asphyxia led to the development of epilepsy. Finally, structural brain alterations were examined.

Results: The animals showed impaired spatial learning and memory and increased anxiety when tested at an age of 3–14 months. Video-EEG at ~10 months showed an abundance of spontaneous seizures, which was paralleled by neurodegeneration in the hippocampus and thalamus, and by aberrant mossy fiber sprouting.

Significance: The present model of birth asphyxia recapitulates several of the later life consequences associated with human HIE. This model thus allows evaluation of the efficacy of novel therapies designed to prevent HIE and seizures

following asphyxia, and of how such therapies might alleviate long-term adverse consequences.

1 | INTRODUCTION

Birth asphyxia (BA; also known as perinatal asphyxia) is a common cause of hypoxic–ischemic encephalopathy (HIE), which is characterized by clinical and laboratory evidence of acute or subacute brain injury, often associated with neonatal seizures.[1,2] BA is the most common cause of death and disability in human neonates and often leads to poor later life outcome, including persistent motor, sensory, and cognitive impairment, behavioral alterations, and epilepsy, characterized by spontaneous recurrent seizures (SRS).[3–5] At least in part, these later life consequences of perinatal asphyxia are thought to be a consequence of asphyxia-induced brain damage. Asphyxia is a global insult on the whole organism,[6] and in the brain, it affects some regions more than others, including the cerebral cortex, hippocampus, basal ganglia, thalamus, and brain stem nuclei.[3–5,7] Despite the high disability burden associated with surviving neonatal HIE in patients, the only evidence-based therapy that is available to reduce BA-induced mortality and brain injury is moderate post-asphyxial hypothermia.[5,8] However, therapeutic hypothermia can only be used to treat HIE in full-term infants, in whom it has a protective effect in only approximately one of six individuals, and survivors remain at high risk for a wide spectrum of neurodevelopmental abnormalities as a result of residual brain injury, highlighting the need for additional, novel therapies to be used in conjunction.[5,8] There is an ongoing debate on whether asphyxia-induced neonatal seizures may exacerbate HIE-induced brain injury and its long-term consequences.[2,9–11] Notably, these seizures are often not sufficiently suppressed by hypothermia,[9] thus necessitating the development of more effective seizure-suppressing therapies.[2,10,12]

To develop novel therapies, animal models of BA and neonatal seizures that mimic the mechanisms of HIE and its clinical short- and long-term consequences are essential.[5,13] The widely used Rice–Vanucci model of hypoxia–ischemia is invasive, based on unilateral ligation of one of the carotid arteries and subsequent exposure to hypoxia.[5] In another popular model, intact neonatal rodents are exposed to hypoxia-only.[14] In both types of models, hypoxia triggers seizures that already commence during the insult,[5,13,14] whereas neonatal seizures in humans usually occur within the first 2–24 h after BA.[1,15] Here, it should be noted that asphyxia is a combination of a decrease in systemic O_2 (hypoxia) and an increase in CO_2 (hypercapnia), and these two components of asphyxia have distinct—often functionally opposite—actions on the physiology and excitability of the neonatal brain.[16,17] Recently, we have described the first noninvasive rat model of BA with seizure generation after, not during, the insult.[17] In this model, intermittent asphyxia is induced at postnatal day 11 (P11), which in terms of cortical development corresponds to term human babies.[18,19] The present study aimed to evaluate (1) the long-term outcome in this model as seen in later life motor, behavioral, cognitive, and structural brain alterations; and (2) whether and how these alterations resemble the clinical situation. Furthermore, we examined whether the animals developed epilepsy in the 14 months after asphyxia examined in this study.

Key Points

- Birth asphyxia often leads to HIE and neonatal seizures, which are important causes of an adverse neurodevelopmental outcome
- Current therapies do not prevent this outcome in the majority of the patients
- Rodent models of HIE and neonatal seizures reflecting the clinical syndrome and its adverse outcome are needed to develop more effective therapies
- Here, we used a novel rat model of birth asphyxia with neonatal seizures and characterized later life motor, cognitive, and behavioral impairments
- We found persistent cognitive and behavioral impairment, epilepsy, and structural brain alterations resembling the outcome in human neonates

2 | MATERIALS AND METHODS

Details on animals and all experimental techniques and statistical methods are described in Appendix S1. In short, the experiments were carried out in male and female P11 Wistar Han rats that were bred in our laboratory. As in our previous experiments,[20] intermittent asphyxia was induced for 30 min by exposing male and female P11 rat pups to three 7 + 3-min cycles of 9% and 5% O_2 at constant

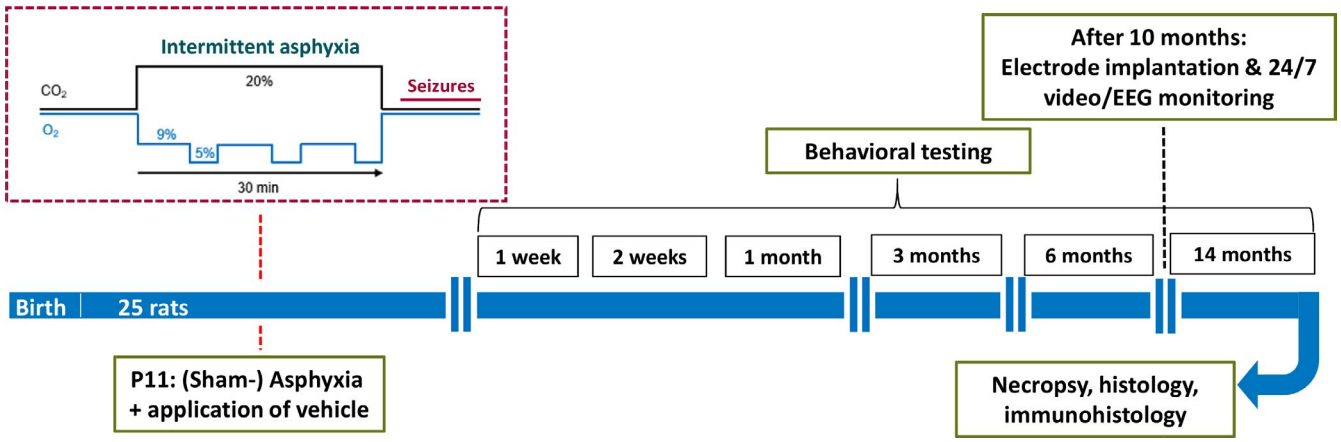


FIGURE 1 Timeline of the experiments on long-term consequences of asphyxia. Approximately 3 weeks following electrode implantation at 10 months after asphyxia, video-electroencephalographic (EEG) monitoring was performed for 1 week. The last behavioral testing was done 14 months after asphyxia to exclude that the anesthesia used for electrode implantation or the video-EEG monitoring affected the outcome of the behavioral experiments

20% CO_2. Sham asphyxia was used for comparison. As shown in Figure 1, a set of behavioral tests was used over 14 months following asphyxia, that is, a large part of the rat's life span. The tests included a modified Irwin test; a test of developmental motor responses; the chimney and rotarod tests to study motor function; the adhesive-removal test to analyze sensorimotor skills; the open field, elevated plus maze and light–dark box tests to determine anxiety-related behavior; and the radial-arm water maze (RAWM) test of spatial learning and memory. Ten months after asphyxia, video-electroencephalographic (EEG) monitoring was used to determine whether asphyxia led to the development of epilepsy. Finally, at the end of the experimental period, structural brain alterations were examined histologically.

3 | RESULTS

3.1 | Behavioral alterations during and after asphyxia in P11 rats

A total of 25 P11 rat pups (12 rats for sham asphyxia and 13 rats for asphyxia) of both sexes were used for the present experiments. Additional P11 rat pups were used for control of body temperature (see Appendix S1). As described recently,[17,20] exposing the P11 rat pups to intermittent asphyxia did not produce any obvious behavioral response, apart from brief agitation on initiation of the exposure. Seizures were never seen during asphyxia. However, in line with previous work,[17,20] following establishment of normocapnic conditions, all rat pups exhibited convulsive seizures, with a latency to the first seizure of approximately 1.2 min on average (range = .7–2.3 min). These seizures, which often had a focal onset, occurred repeatedly over 5.2 min (range = 1.3–8.8 min) and ended 6.3 min (range = 2.2–9.9 min) after the asphyxia. Subsequently, all animals resumed apparently normal behavior. Seizures were scored by a modified Racine scale[20] as shown in Figure S1 and described in Appendix S1. In agreement with previous findings,[17,20] the predominant seizure type was generalized convulsive (Stage III–V) seizures seen in all animals, whereof generalized tonic–clonic (Stage V) seizures were observed in 9 of 13 rat pups (Figure S2). No animal died during or after asphyxia, and no sex differences were observed. Rectal body temperature was similar before and after asphyxia and not different from sham controls (Table S1).

3.2 | Body weight gain after asphyxia

As shown in Figure S3, body weight gain in male and female rats did not differ between animals with asphyxia and those with sham asphyxia.

3.3 | Estrous cycle after asphyxia

Because cognitive and anxiety functions may be affected by the female estrous cycle,[21] we determined whether the postasphyxial female rats had regular estrous cycles. As shown in Table S2, at approximately 6 months after asphyxia or sham asphyxia, all female rats exhibited a regular estrous cycle without any obvious difference between sham controls and postasphyxial rats. The estrous cycle was not synchronized among the individual animals (Table S2).

3.4 | Long-term motor and behavioral consequences of asphyxia with neonatal seizures

Much of the full behavioral repertoire of rats is observed only in adolescence and adulthood.[22,23] Thus, in contrast to previous studies in other rat models, which evaluated consequences of HIE or hypoxia-only over a few days or weeks after the insult,[5,13,14] we evaluated the behavioral consequences of asphyxia with neonatal seizures over 14 months, that is, a large part of the rat's life span. As shown in Figures 1 and S4, the two groups of rats, that is, the sham group ($n = 12$; six males, six females) and the asphyxia group ($n = 13$; seven males, six females) were repeatedly tested for behavioral alterations. Figure S4 summarizes the outcome of the various tests, which are illustrated in Figure S5. Males and females are separately shown in all figures, and sex differences are noted in figure legends.

3.4.1 | Developmental motor responses at P18

Tests of developmental motor responses (see Figure S5A), which were performed only once (at 7 days after asphyxia), were not affected in the postasphyxia group (Figure S4).

3.4.2 | Modified Irwin test at P18 to 14 months

As shown in Figure S4, the modified Irwin test (see Figure S5A) did not indicate any obvious intergroup differences in general behavior at any time after asphyxia.

3.4.3 | Chimney test at P22

In the chimney test, neurological deficit is indicated by the inability of the animals to climb backwards through a tube within 30 s. As shown in Figure S6, all rats of all groups were able to do this.

3.4.4 | Rotarod at P19 to 14 months

In the rotarod test, which was repeatedly performed over 14 months following asphyxia (Figure S4), not all rats were capable (or motivated) of staying on the rotating rod for 60 s despite previous training, but there was no significant difference between groups (Figure S7). Over the 14 months after asphyxia, both groups improved their performance on the rod.

3.4.5 | Sensorimotor function (adhesive-removal test) at P25 to 14 months

In this test, neither time to first contact with the adhesive tape (see Figure S5D) nor time from the first contact to the removal of the tape was different between sham-treated and postasphyxial rats (Figure S8). In both experimental groups, animals improved over the experimental period.

3.4.6 | Tests for anxiety-related behavior at P18 to 14 months

Although measurements of anxiety-related behavior in rodents are often done using a single test, experience from neuropharmacological work has shown it is better to use several kinds of tests that measure anxiety under different conditions.[24,25] Here, three unconditioned response tests (which require no training and usually have a high eco-/ethological validity) were used: the open field test (P18 to 14 months), the elevated plus maze test (P26 to 14 months), and the light–dark box test (P27 to 14 months). All three tests are based on the conflict of rodents between exploration and natural aversions to illuminated, open, and/or elevated areas.[26] Thus, as shown in Figure S9 for the open field test, rats stayed much longer in the outer zone of the field compared to the aversive middle and center zones, without any significant intergroup difference. Furthermore, both groups exhibited the same explorative activity as indicated by the distance moved and velocity.

Similarly, in the elevated plus maze test (Figure S10), rats stayed longer in the closed arms than in the center or open arms of the maze, without any significant intergroup difference. Furthermore, both groups exhibited the same explorative activity as indicated by the distance moved and velocity (Figure S4). However, it should be noted that in both open field (Figure S9) and elevated plus maze (Figure S10), the time spent in the aversive locations of these tests was already quite low in sham controls, which makes it difficult to determine an increase in anxiety-related behavior in the postasphyxial rats.

In the light–dark box test, significantly increased anxiety-related behavior was observed in postasphyxial rats at 6 and 14 months after asphyxia. As shown in Figure 2, sham controls stayed a much shorter period of time in the aversive light compartment of the test than in the dark compartment, which did not change over the 14 months of the trial period. Both the duration of stay in the light

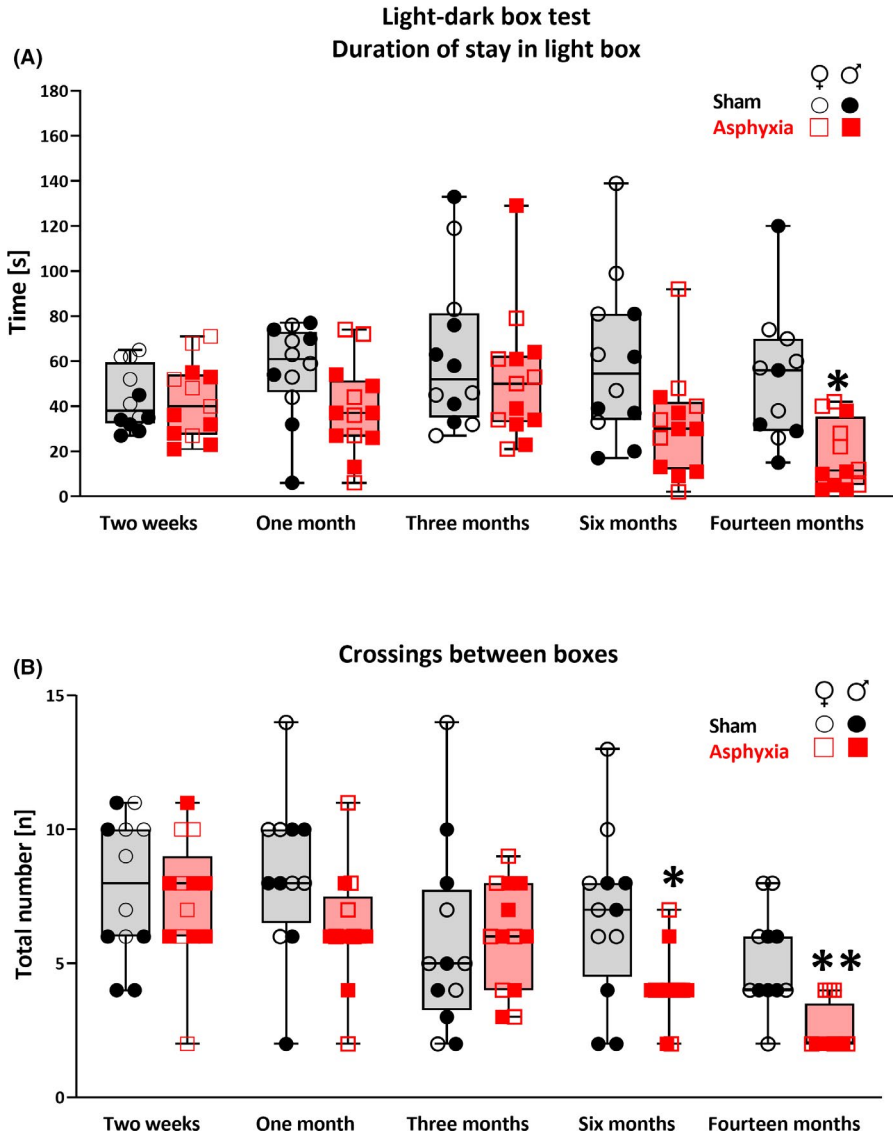


FIGURE 2 The behavior of rats in the light–dark box test at different developmental or time periods following asphyxia. As described in Appendix S1, time spent in each compartment, and the crossings between compartments, were measured for 3 min by a video tracking system. Data are shown as boxplots with whiskers from minimum to maximal values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. Male and female rats are shown by different symbols (see key in the figure). For each period, the duration of stay in the aversive light compartment and the number of crossings between the light and dark compartments are shown. Data were analyzed with a two-way analysis of variance (ANOVA) mixed-effects model (see Appendix S1), followed by Sidak's multiple comparisons test. For duration of stay in the light compartment, results from two-way ANOVA were $F_{3.153,\ 70.95} = 3.084$ for time ($p < .05$) and $F_{1,\ 23} = 10.3$ ($p < .01$) for column factor. For crossings between compartments, results from two-way ANOVA were $F_{1.934,\ 42.55} = 26.47$ for time ($p < .00001$) and $F_{1,\ 23} = 12.55$ ($p < .01$) for column factor. Within each group, sham controls did not significantly differ across the five periods, whereas postasphyxial rats stayed for a significantly shorter time in the light box at 14 months after asphyxia and exhibited fewer crossings between the dark and light box at 6 and 14 months compared to 2 weeks, indicating the development of anxiety-related behavior. These intragroup differences are also mirrored by significant differences from sham controls, which are indicated by asterisks (*$p < .05$; **$p < .01$). No significant sex differences were observed

compartment and the crossing between the dark and light compartments were significantly reduced in postasphyxial rats, indicating increased anxiety-related behavior. Interestingly, the data in Figure 2 indicate that this behavior of postasphyxial rats developed slowly, with significant effects only reached at 6–14 months after the insult.

3.5 | Long-term cognitive alterations at 3 and 6 months after asphyxia with neonatal seizures

Alterations in learning and memory were determined by the RAWM.[27] The RAWM is a hybrid of the Morris water

maze and a radial-arm maze (Figure S5H), which takes advantage of the simple motivation provided by immersion into water, together with the benefits of scoring errors (rather than time or proximity to platform location) associated with the radial-arm maze.[27] At 3 and 6 months after asphyxia, sham controls exhibited significant learning on the second day of the test (Figure 3A,B) in the 2-day reference memory version of this task (Figure S5H; see Appendix S1). Learning was not observed in the asphyxia group, indicating that asphyxia with neonatal seizures induce long-term deficits in reference memory or other cognitive processes required for the task. It is worth mentioning that after the training period (Trials 1–12, Day 1), all rats of both groups were able to find the platform and almost all trials were completed successfully within 60 s (sham: 3 months = 98.1%, 6 months = 100%; asphyxia: 3 months = 97.9%, 6 months = 99.5%). However, as shown in Figure 3C,D, the postasphyxial rats made significantly more errors in finding the correct arm of the maze, and this did not improve throughout the experiment.

Apart from that, the described deficits were also observed just by analyzing the rat´s exploration strategies. Both groups started to systematically search for the platform in one arm after another; however, at the end of the task, most of the sham-controls swam into the center and turned around for better orientation to directly choose the goal arm. In contrast, post-BA rats stuck to the systematic searching approach described before, thus finding the goal arm by chance after several errors. In addition, they sometimes did not swim to the very end of one arm but rather turned around before reaching a possible platform location, which could reflect enhanced impulsivity. Figure 3C,D summarizes the cumulative errors recorded in the two groups of rats, substantiating the marked cognitive impairment of the asphyxia group.

3.6 | Development of spontaneous recurrent seizures after asphyxia with neonatal seizures (recorded at ~10.5 months of age)

As described in Appendix S1, two of the 25 rats (one male sham rat, one male postasphyxial rat) had to be euthanized at ~6 and 9 months, respectively. The remaining 23 animals (11 shams, 12 asphyxia) were implanted with EEG electrodes at 10 months following asphyxia (Figure 1; Figure S11) to avoid the insult produced by electrode implantation from affecting the behavioral and cognitive alterations after asphyxia. Continuous (24/7) video-EEG monitoring for 1 week started ~3 weeks after the electrode implantation. A total of 3864 h of video-EEG recording in 23 rats was visually analyzed for the occurrence of spikes, spike clusters, electrographic or electroclinical seizures, and other abnormal epileptiform activity by two experienced observers, who were not aware of whether the rats were from the sham or asphyxia groups. In case of any abnormal EEG activity, the concomitant video was viewed for behavioral alterations. Based on the commonly used definition of seizures,[28] a seizure was defined as paroxysmal EEG alteration consisting of epileptiform spikes and sharp wave trains with an amplitude at least two times greater than the background, a frequency of at least 2 Hz, and a duration of at least 5 s. Three types of SRS were found: (1) electrographic seizures without any obvious behavioral alteration in the concurrent videos (Figure 4B); (2) nonconvulsive electroclinical seizures (Figure 4C,D) that had an associated behavioral correlate, such as sudden behavioral arrest, staring episodes, head-jerking, and facial automatisms; and (3) generalized convulsive electroclinical seizures (Figure 4E). As shown in Figure 4, the EEG alterations during these three seizure types were polymorphic. Examples of such EEG seizures and their behavioral correlates are shown in Video S1.

SRS were recorded in all postasphyxial rats (Figure 5A). Median SRS frequency was 92/week, with a wide interindividual range from four to 1213/week. As shown in Figure 5C,D, electrographic seizures were more frequent than electroclinical seizures. Most electroclinical seizures were nonconvulsive; only one postasphyxial rat exhibited generalized convulsive seizures with loss of righting reflexes and clonic movements of both hindlimbs. The occurrence of the SRS over the 7 days of the recording period is illustrated in Figure S2. Most SRS had a duration of 5–10 s (please note the lower limit of duration was set at 5 s). In addition to these seizures, spikes and spike clusters with a duration of 2–4 s were frequently observed (not illustrated).

However, some epileptiform EEG alterations were also observed in approximately one third of the sham control rats (Figure 5A). They occurred at much lower frequencies than in the postasphyxial rats (Figures 5B–D, S2). Furthermore, none of the sham controls exhibited generalized convulsive seizures. The presence of these rare low-frequency epileptiform discharges in control rats is consistent with previous reports[29] and may be either genetically inherent or a consequence of the brain injury in response to the EEG electrode implantation (see Section 4). As described in Appendix S1, no obvious histological alterations were observed in the cortex below the epidural screw electrodes in any rat. Regardless, both the incidence and frequency of SRS were much higher in postasphyxial rats than in the controls (Figure 5). The background EEG (Figure 4A) did not show obvious differences between the control and postasphyxial rats.

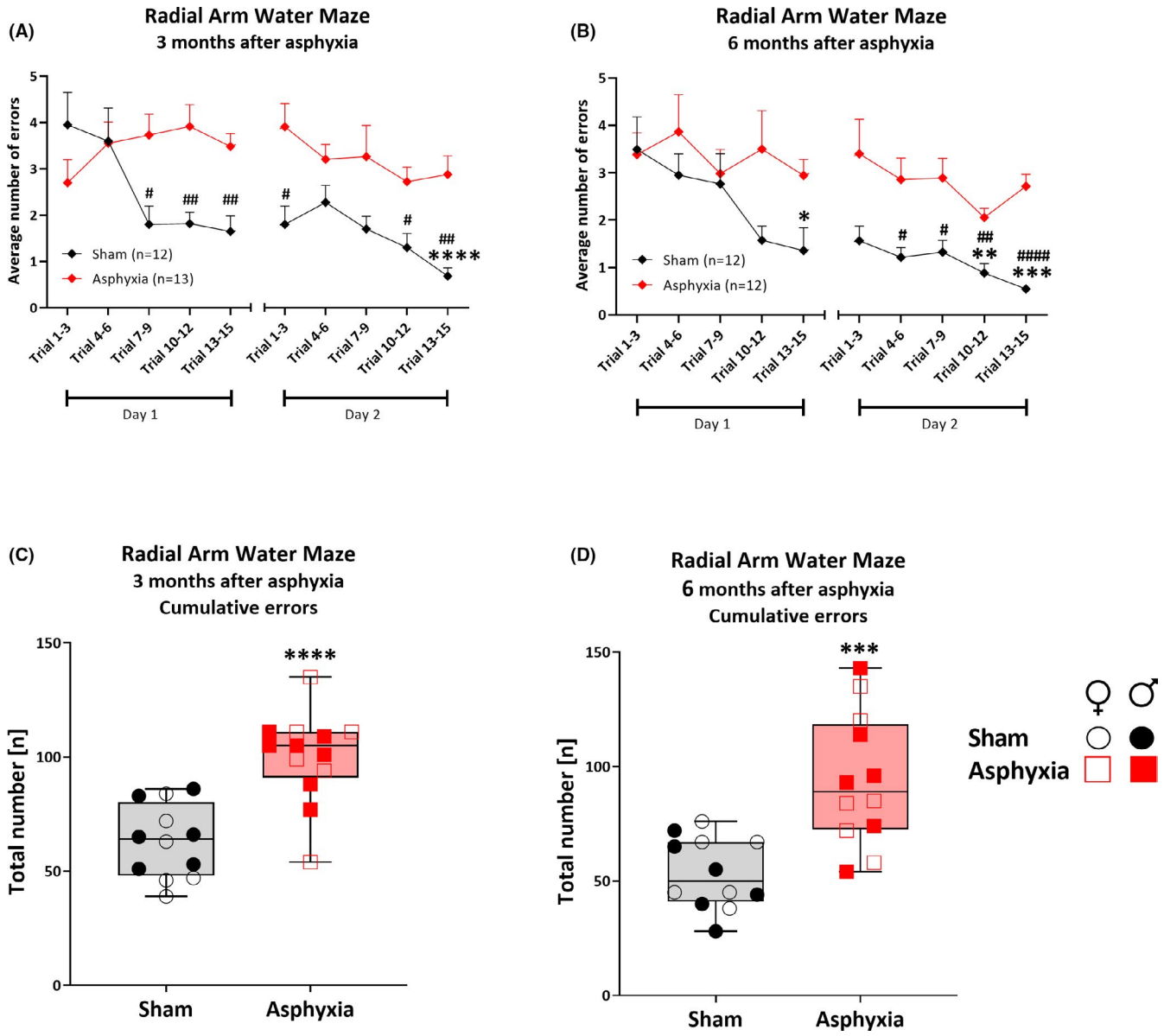


FIGURE 3 Performance of rats in the radial-arm water maze test. The test was repeatedly performed over 2 days at 3 months (A, C) and 6 months (B, D) following asphyxia. In A and B, data are shown as mean ± SEM. In C and D, data are shown as boxplots with whiskers from minimum to maximal values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. In A and B, data from both sexes were poooled, whereas in C and D, male and female rats are shown by different symbols (see key in D). For comparison of different trials within one group, the repeated measures one-way analysis of variance (ANOVA) test for nonparametric data (Friedman test), followed post hoc by Dunn's multiple comparison test was used. For comparing data of sham- and asphyxia-treated animals, the two-way ANOVA test followed post hoc by Sidak's multiple comparisons test was used. Group differences in cumulative errors were analyzed by the *t*-test. (A) The average number of errors in sham controls and rats that had experienced asphyxia and neonatal seizures 3 months before the radial-arm water maze test. The Friedman test indicated significant differences within each group for sham rats (Q value = 33.97, $df = 9$, $p < .0001$) but not postasphyxial rats (Q value = 10.87, $df = 9$, $p = .285$). Two-way ANOVA indicated significant differences between both groups: $F_{5.7,\ 131.1} = 3.194$ ($p = .0067$) for time and $F_{1,\ 23} = 26.6$ ($p < .0001$) for column factor. Data from post hoc analysis indicating significant learning within the sham group (vs. Trials 1–3) are indicated by asterisks. Significant differences between both groups are indicated by hash signs. (B) The same as A but 6 months after asphyxia. The Friedman test indicated significant differences within each group for sham rats (Q value = 39.14, $df = 9$, $p < .0001$) but not postasphyxial rats (Q value = 4.687, $df = 9$, $p = .8607$). Two-way ANOVA indicated significant differences between both groups: $F_{4.63,\ 101.9} = 4.701$ ($p = .0009$) for time and $F_{1,\ 22} = 16.15$ ($p = .0006$) for column factor. (C) Cumulative errors within each group over the whole duration of the 30 individual trials (2 days) at 3 months after asphyxia. Significant differences from sham controls are indicated by asterisks. (D) Cumulative errors within each group over the whole duration of the 30 individual trials (2 days) at 6 months after asphyxia. Significant differences from sham controls are indicated by asterisks. There were no significant differences between male and female rats. #$p < .05$, ##$p < .01$, ####$p < .00001$, *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$

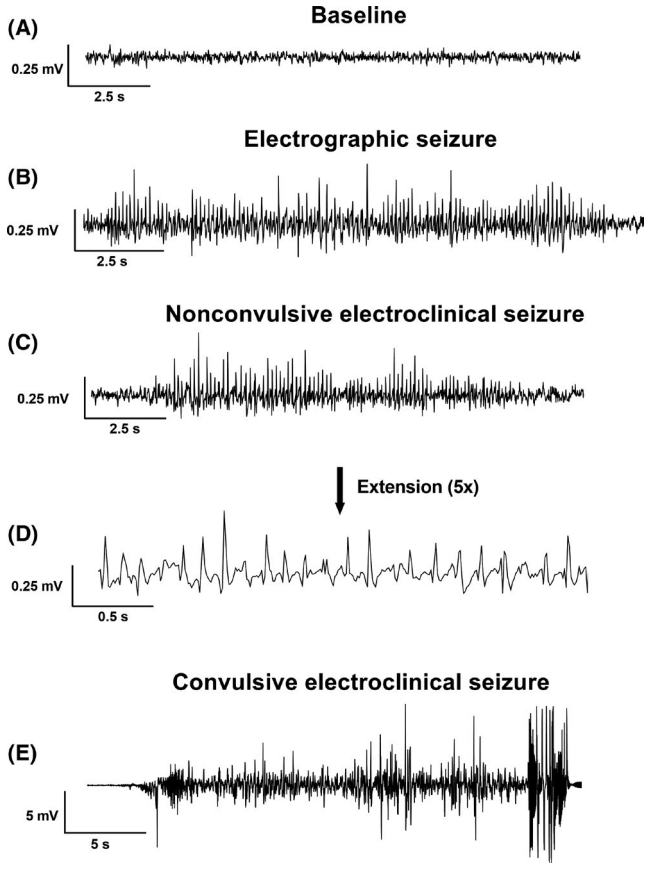


FIGURE 4 Representative electroencephalographic (EEG) recordings of rats at ~10.5 months after asphyxia. The EEG was recorded with the epidural screw electrodes located above the motor cortex close to the hippocampal formation. (A) Normal baseline (background) EEG. (B) An electrographic seizure that was not associated with any obvious behavioral abnormality in the corresponding video. However, subtle behavioral abnormalities may have been missed in the video. (C) A nonconvulsive electroclinical seizure. During the paroxysmal EEG activity, a behavioral arrest was observed in the corresponding video. (D) shows an extended part (5×) of the seizure illustrated in C. (E) A generalized convulsive electroclinical seizure. Note the much higher amplitude of the paroxysmal EEG activity. During the paroxysmal EEG activity, the loss of righting reflexes and clonic movements of both hindlimbs were observed in the corresponding video

3.7 | Neurodegeneration in hippocampus and thalamus, and aberrant mossy fiber sprouting in postasphyxial rats (determined at ~14 months of age)

Survivors of HIE are at increased risk of cognitive impairment, which is thought to be at least in part a consequence of hippocampal damage.[7,30] Thus, the deficit in spatial learning and memory observed following asphyxia with neonatal seizures in the RAWM indicated that affected rats might exhibit neurodegeneration in the hippocampus, which plays a crucial role in spatial memory.[31] Given the functional differences between the dorsal and ventral hippocampus,[32–34] we first examined sections of the dorsal hippocampus, which is involved with cognitive and memory functions in rats. As shown in Figure 6A, the dorsal hippocampal formation of the two groups of rats displayed normal features when NeuN- and DAPI-stained sections were compared, but at higher magnification (Figure 6B) differences in neuronal density can be seen. Quantification of NeuN-positive neurons in the dentate hilus demonstrated a significant neuronal loss in postasphyxial rats (Figure 7A). No significant intergroup differences were obtained for the area of the hilus (Figure 7B). In addition to the cell loss in the dentate hilus, a significant reduction of neurons in CA3c was determined in postasphyxial rats (Figure 7C). In contrast, no obvious neurodegeneration was observed in CA1 of the dorsal hippocampus (Figure 7D).

In addition to the dorsal hippocampus, we examined sections of the ventral hippocampus, which is involved in affective processes such as anxiety in rodents.[33,34] As shown in Figure S12, findings were similar to those in the dorsal hippocampus, with significant loss of neurons in dentate hilus and CA3c.

The dentate hilus is a polymorphic layer with two major cell types, glutamatergic mossy cells and diverse γ-aminobutyric acidergic (GABAergic) interneurons, which exhibit different vulnerabilities to brain injuries.[35] This prompted us to differentiate mossy cells and different subtypes of GABAergic interneurons by immunohistochemistry (Figure S13). As shown in Figure S14A,C, in both dorsal and ventral hippocampus, mossy cells and parvalbumin-positive GABAergic interneurons were preferentially lost after asphyxia, whereas no significant loss of somatostatin-positive GABAergic interneurons was observed. We also determined the percentage of the three subpopulations of hilus neurons of all NeuN-positive neurons counted in the hilus, confirming that the mossy cells and somatostatin-positive interneurons were the major cell types in the hilus and illustrating the effect of asphyxia (Figure S14B,D).

Loss of mossy cells in the dentate hilus may lead to aberrant sprouting of mossy fibers.[36] Granule cell axons (mossy fibers) project into the hilus of the dentate gyrus and stratum lucidum of CA3 in rodents and other species, including humans, where they synapse with hilar mossy cells, inhibitory interneurons, and CA3 pyramidal cells, but only very rarely with other granule cells.[36] Accordingly, when synaptoporin was used to label mossy fibers in the dorsal hippocampus, the highest density of labeling was observed in the dentate hilus and stratum lucidum of CA3 (Figure 6C). In postasphyxial rats, aberrant mossy fiber sprouting (MFS) was observed in the stratum

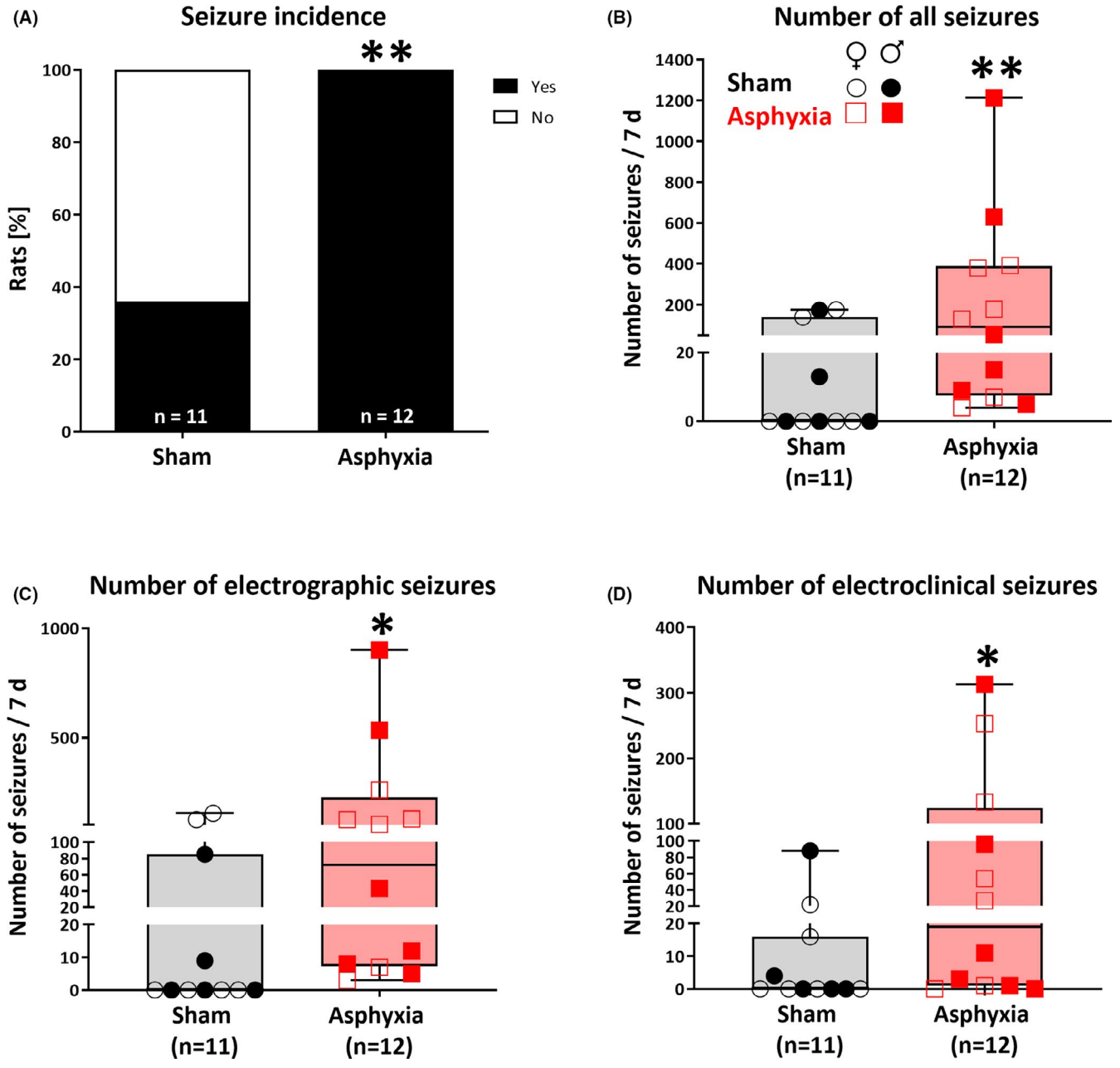
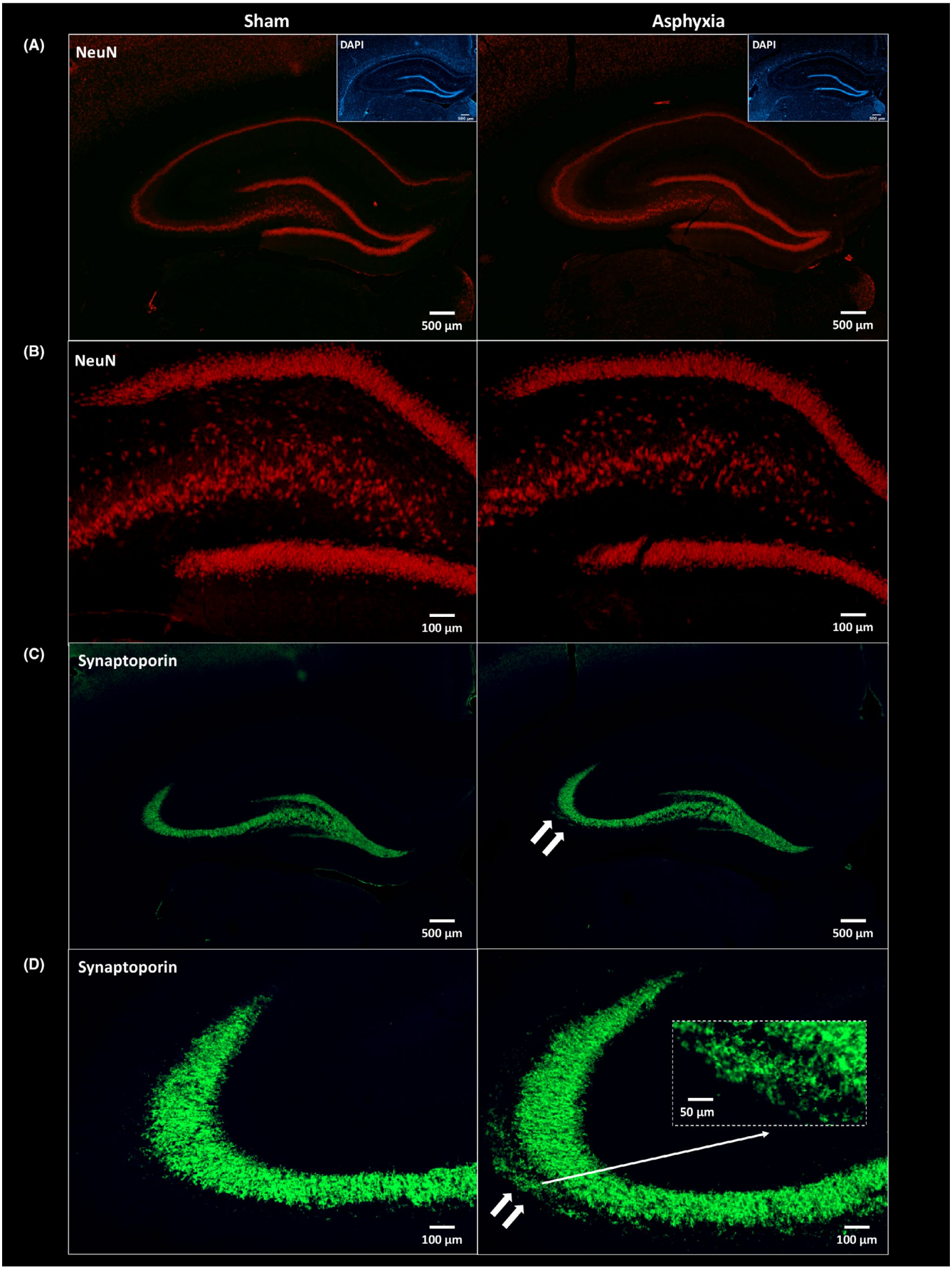


FIGURE 5 Incidence and frequency of electrographic and electroclinical seizures in rats at ~10.5 months after asphyxia. Because paroxysmal activity was also observed in sham controls (but with much lower incidence and frequency), this is shown in comparison. Group differences were analyzed by the Barnard test (seizure incidence) or the Mann–Whitney test (seizure frequencies). Significant differences between sham controls and postasphyxial rats are indicated by asterisks (*$p < .05$; **$p < .01$). Data in B–D are shown as boxplots with whiskers from minimum to maximal values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. In A, data from both sexes were poooled, whereas in B–D, male and female rats are shown by different symbols (see key in B). (A) Percent incidence of all seizure types in sham controls and postasphyxial rats. (B) The number of all seizures recorded by continuous (24/7) monitoring in 1 week. (C) Number of electrographic seizures/week. (D) Number of electroclinical seizures/week. Note that the majority of these seizures were nonconvulsive (see text). Only paroxysmal electroencephalographic events with a duration of at least 5 s were considered seizures. No sex differences were observed

FIGURE 6 Representative photomicrographs of sections of the dorsal hippocampus from sham controls and postasphyxial rats at ~14 months after asphyxia. (A) Overview of the left dorsal hippocampus illustrated by NeuN- and DAPI-stained sections. (B) NeuN-stained sections at higher magnification. Note the obvious cell los in the dentate hilus and CA3c region of postasphyxial rats. (C) Synaptoporin-stained sections. (D) Synaptoporin-stained sections at higher magnification to illustrate the aberrant mossy fiber sprouting into the stratum oriens of the CA3 region (arrows). For better visualization, a twofold enlargement of this region of interest was inserted into the bottom right picture.


Sham
Asphyxia
(A)
NeuN
DAPI
500 µm
(B)
NeuN
100 µm
(C)
Synaptoporin
500 µm
(D)
Synaptoporin
50 µm
100 µm

oriens of the CA3 hippocampal region (Figure 6C,D). Quantification of these data by image analysis is shown in Figure 7E. In contrast to post-status epilepticus models of temporal lobe epilepsy,[36] no aberrant MFS was observed in the inner molecular layer of the dentate gyrus (Figure 6C), which is similar to findings in hypoxia-only models of HIE.[14]

As described above, aberrant MFS is often a consequence of loss of hilar neurons[36] as observed here. Thus, we examined the correlation between the hilar neuron density and MFS in the 23 rats of the two groups used for this analysis. As shown in Figure 7F, a significant correlation coefficient ($r = -.7074$, $p = .0002$) was obtained, indicating that the most marked MFS occurred in rats with the highest loss of hilar neurons.

In addition to the hippocampus, NeuN-stained sections of several other brain areas, including the cerebral cortex, globus pallidus, caudate–putamen, subthalamic nucleus, and different thalamic areas, were visually examined for any obvious neuronal damage. These brain areas were chosen based on clinical findings following asphyxia[3–5,7] as described in Section 1 and on findings in hypoxia–ischemia models of HIE.[5,13] Differences between sham controls and postasphyxial rats were present in thalamic areas below the third ventricle, that is, the paraventricular and mediodorsal thalamus (Figure S15). Quantification of the density of NeuN-positive cell bodies demonstrated significant neuronal loss in the postasphyxial rats (Figure S16).

3.8 | Correlation between seizure frequency and structural brain alterations

In Figure S2, individual seizure frequencies, neuronal loss in the hilus, and aberrant MFS are illustrated for all 23 rats that were finally used for video-EEG monitoring. Nonparametric Spearman correlation analysis showed that both neuronal loss in the hilus and MFS were significantly correlated with the frequency of SRS (Figure S17). The correlation coefficient r was $-.6667$ for hilar neuronal density versus SRS frequency ($p = .0005$) and .4770 for intensity of MFS versus SRS frequency ($p = .0214$).

4 | DISCUSSION

This is to our best knowledge the first study that describes the long-term consequences of asphyxia (not pure hypoxia) and subsequent neonatal seizures in a rodent model. Rodent models of HIE/neonatal seizures used in previous work were either hypoxia–ischemia models or hypoxia-only models,[14,15] which lack the respiratory acidosis (hypercapnia) that is a fundamental constituent of BA.[37] Hypercapnia has numerous effects on brain functions and neuronal excitability.[16,17] One of the striking differences between hypoxia–ischemia or hypoxia-only models and the present asphyxia model is that seizures start during hypoxia in the former.[13,14] This is in line with in vitro data showing that hypoxia as such promotes neuronal excitability and synaptic potentiation.[38,39] Moreover, the highly artificial condition of pure hypoxia in vivo (which never occurs during cardiorespiratory failure or local/global ischemia under nonexperimental conditions) leads to a brain alkalosis that further promotes excitability (for data and references, see Pospelov et al.[16] and Ala-Kurikka et al.[17]). In contrast, hypercapnia, that is, an elevation of systemic CO_2, produces a fall in brain pH and a consequent decrease in neuronal excitability (see Ruusuvuori and Kaila[40] and references cited therein), which explains the finding that neonatal seizures do not occur during asphyxia but only during the subsequent establishment of normocapnic conditions and brain pH recovery.[16,17] This is observed both in human neonates and also in relevant large-animal models, in which neonatal seizures are triggered after a period of moderate or severe asphyxia.[5] Thus, the brain is normoxic during neonatal seizures, which is a unique, translationally relevant feature of the present model compared to all rat and mouse hypoxia–ischemia or hypoxia-only models. Notably, the present data indicate that the differences in the mechanisms involved in HIE and seizure generation affect later life outcome, which obviously is an important aspect of translational research of this kind.

In Table 1, the observations in our rat model of BA are compared with those obtained in hypoxia-alone, hypoxia–ischemia, and ischemia rat and mouse models of HIE and neonatal seizures. The only commonality across most models is the development of epilepsy, with SRS and aberrant MFS in the hippocampus. The lack of any obvious hippocampal damage in rat hypoxia-only models, which is in sharp contrast to the consequences of HIE in humans,[5] may explain why no consistent cognitive impairment has been reported in these models, whereas such impairment is seen in all other models in Table 1.

A point worth emphasizing in the present context is the developmental stage of brain development of rat and mouse pups used in the various models. Traditional models of developmental brain injury have utilized rodents at P7, which, based on numerous milestones of cortical (i.e., hippocampal and neocortical) development, corresponds to a preterm neonate with a mean of the estimated postconceptional age of approximately 25–30 weeks.[41,42] In contrast to this, rats and mice at the age of P10–P12 exhibit many of those developmental brain characteristics that are achieved by the human term neonate (i.e., when

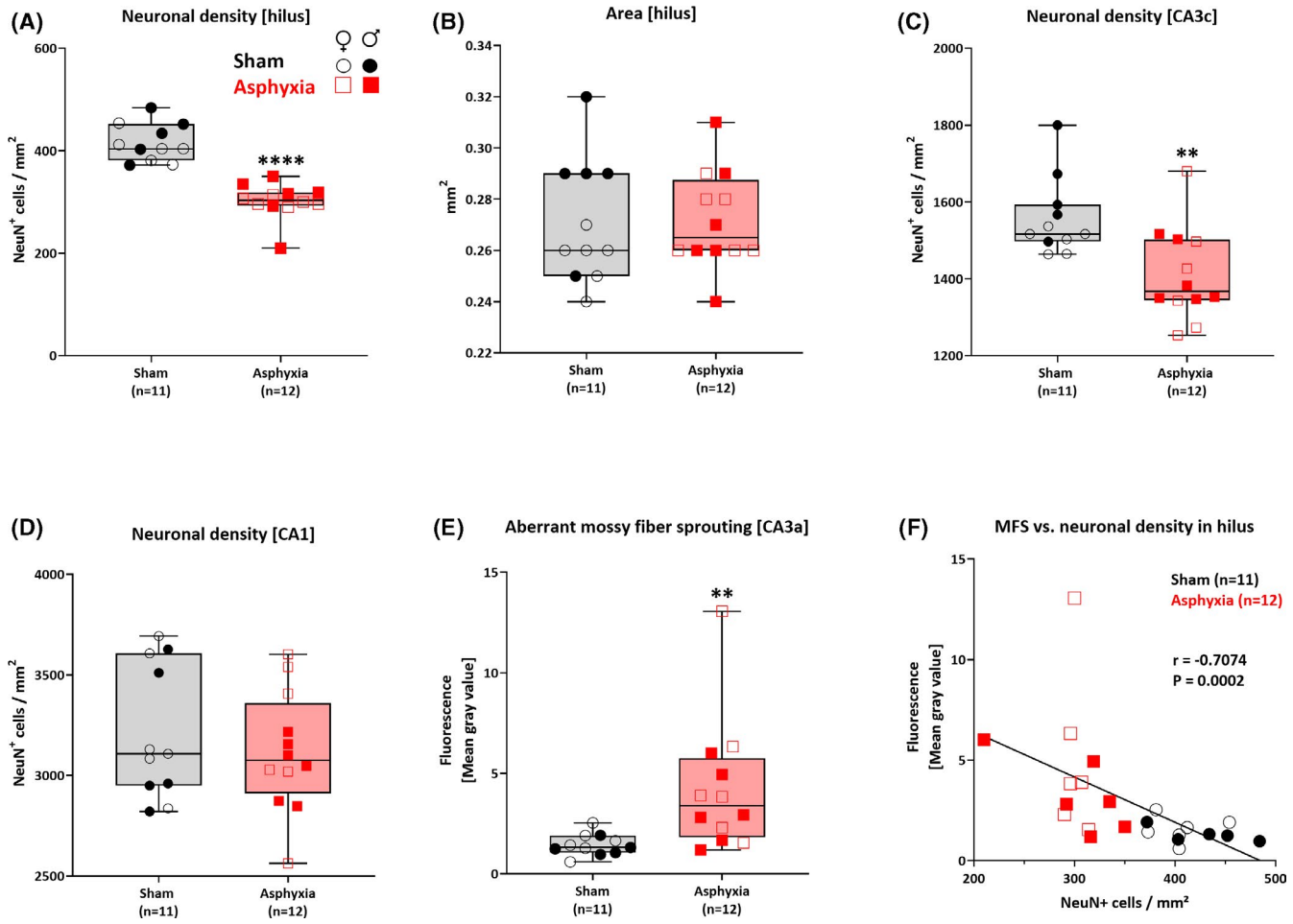


FIGURE 7 Neurodegeneration and aberrant mossy fiber sprouting (MFS) in the dorsal hippocampus of postasphyxial rats, determined ~14 months after asphyxia. Data in A–E are shown as boxplots with whiskers from minimum to maximal values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. Male and female rats are shown by different symbols (see key in A). Group differences were analyzed by the Mann–Whitney test. Significant differences between sham controls and postasphyxial rats are indicated by asterisks (**$p < .01$; ****$p < .0001$) and were determined from six hippocampi with three slices taken from each animal at section levels −3.0, −3.16, and −3.32 mm from bregma. (A) Neuronal density in the hilus. (B) Average area of the hilus. (C) Neuronal density in CA3c. (D) Neuronal density in CA1. (E) Aberrant MFS into the stratum oriens of the CA3. (F) Correlation between MFS and neuronal density in the hilus. Note that all rats that exhibited MFS had lower neuronal densities than sham controls; the higher the neuronal loss, the higher the MFS ($r = -.7074$; $p = .0002$). One postasphyxial rat had much higher MFS than the rest of the group. When excluding this rat as an outlier, the Spearman rank method resulted in a correlation coefficient (r) of −.6927 ($p = .0004$). No significant differences in the long-term effects of asphyxia between male and female rats were detected in any of the data illustrated in this figure. However, the hilus area in male sham rats was significantly bigger than that of female sham rats ($p = .0498$). Furthermore, neuronal density in the CA3c of sham controls was significantly lower in female than male rats ($p = .0455$)

finishing 40 gestational weeks), including continuity and other features of the EEG,[18,19,43] as well as similar cortical expression patterns of important genes/proteins,[44] such as the neuron-specific KCC2, which is an excellent indicator of neuronal maturity.[45,46]

In the present study, the consequences of intermittent asphyxia with neonatal seizures for motor, behavioral, and cognitive functions as well as for brain structure were evaluated over a period of ~14 months after asphyxia, that is, a large part of the life span of the Han:Wistar outbred rats used here, which have a median life expectancy of 30–33 months (females) and 33–36 months (males).[47] Following asphyxia with subsequent seizures, no later life motor or somatosensory abnormalities were observed, but the postasphyxial rats developed increased anxiety, a striking cognitive decline, a high incidence and frequency of SRS, neurodegeneration in the hippocampus and thalamus, and aberrant MFS in the stratum oriens of the CA3 sector of the hippocampus. As shown in Table 1, these alterations resemble the clinical situation, demonstrating

TABLE 1 Comparison of later life consequences in rat and mouse models of HIE and neonatal seizures with such consequences following prolonged birth asphyxia in human neonates

	Rat and mouse models of birth asphyxia/HIE and neonatal seizures				
	Noninvasive models		Invasive models		
Later life consequences	**Intermittent asphyxia [hypoxia and hypercapnia]**	**Hypoxia-alone**	**Hypoxia–ischemia [Rice–Vannucci] models of HIE [with unilateral carotid artery ligation]**	**Ischemia-alone [with unilateral carotid artery ligation]**	**Prolonged birth asphyxia resulting in HIE with neonatal seizures in human neonates[a]**
Species and postnatal age	P11 rat	P8–P12 rat; P7–P8 mouse	P7 rat; P10–P12 rat; P7 mouse	P7–P12 mice (neonatal rats do not exhibit seizures after ischemia-alone)	
Neurodegeneration	Yes (in hippocampus, and thalamus)	No (rat) Yes (in hippocampus of mice)	Yes (gray matter injury observed in cortex, hippocampus, thalamus, and basal ganglia)	Yes (ipsilateral hemispheric and hippocampal atrophy)	Yes (gray matter injury particularly observed in cortex, hippocampus, thalamus, basal ganglia, and brain stem)
Mossy fiber sprouting in hippocampus	Yes	Yes	Yes	N/A	N/A (but seen in adult patients with mesial temporal lobe epilepsy)
Developmental delays	No	N/A	N/A	N/A	Yes
Learning and memory deficits	Yes (impaired spatial learning and memory)	Mixed results	Yes (impaired spatial learning and memory)	Yes	Yes (related to smaller hippocampal volume)
Hyperactivity	No	Yes	N/A	Yes	Yes
Increased aggression	No	Yes	N/A	N/A	Yes
ADHD	NT	Yes	Yes	N/A	Yes
Autistic spectrum disorders	NT	Yes (social deficit)	Yes (reduced attention)	No (but large variation)	Yes
Schizophrenia-like symptoms	NT	Yes	N/A	N/A	Yes
Anxiety	Yes	Yes	Yes	No (but large variation)	Yes
Disturbed sleep	NT	Yes	N/A	Yes	Yes
Impaired sensorimotor skills	No (adhesive removal test)	N/A	Yes	N/A	Yes
Hearing and vision loss	NT	N/A	N/A	N/A	Yes
Epilepsy with SRS	Yes	Yes	Yes	N/A	Yes

(Continues)

TABLE 1 (Continued)

	Rat and mouse models of birth asphyxia/HIE and neonatal seizures				
	Noninvasive models		Invasive models		
Later life consequences	**Intermittent asphyxia [hypoxia and hypercapnia]**	**Hypoxia-alone**	**Hypoxia–ischemia [Rice–Vannucci] models of HIE [with unilateral carotid artery ligation]**	**Ischemia-alone [with unilateral carotid artery ligation]**	**Prolonged birth asphyxia resulting in HIE with neonatal seizures in human neonates[a]**
Motor disabilities	No	N/A	Yes	N/A	Yes
CP and other motor disorders	No	No	No	No	Yes (but more recent work has shown that birth asphyxia does not play a major role in the incidence of CP)
Disadvantages of the model	New model ⇒ only few data on variability across laboratories	High variability across laboratories; hypoxia-only does not replicate birth asphyxia	High variability across laboratories; the invasive nature of severing the common carotid artery does not replicate human injury	Rarely used in the context of neonatal seizures; the invasive nature of severing the common carotid artery does not replicate human injury	–
References	Present study	Sun et al.,[14] Millar et al.,[5] Quinlan et al.,[78] Hamdy et al.[13]	Kadam and Dudek,[75] Kadam et al.,[79] Millar et al.,[5] Hamdy et al.[13]	Kang and Kadam,[77] Kang et al.[80]	de Haan et al.,[30] van Handel et al.,[3] Ahearne et al.,[4] Millar et al.,[5] Korzeniewski et al.[81]

Note: Rodent models in which neonates are asphyxiated in utero are not included, because such models do not allow studying neonatal seizures.[13]

Abbreviations: ADHD, attention-deficit/hyperactivity disorder; CP, cerebral palsy; HIE, hypoxic–ischemic brain injury; N/A, no data found; NT, not tested yet; P, postnatal day; SRS, spontaneous recurrent seizures.

aThe long-term effects of birth asphyxia depend on the age at the time of insult (which also has a major impact on which region of the brain is injured) and the severity of the injury; there is a critical threshold of asphyxia beyond which brain damage occurs[82]; furthermore, outcomes are poorer for those with more severe asphyxia.[30]

that the present model recapitulates several of the later life consequences of BA in human neonates. No obvious sex differences were observed in most readouts; however, such comparison between sexes was affected by the relatively low sample size of the male and female subgroups.

Interestingly, the increased anxiety-related behavior observed in the light–dark box developed slowly; that is, significant differences from sham controls were seen only after 6–14 months. Thus, this behavior would not have been detected by a shorter observation period or the use of only one anxiety model, as done in most previous studies using other models of BA/HIE and neonatal seizures (Table 1). The present finding of the protracted development of anxiety after asphyxia matches strikingly with what has been observed at an age of 38 years in the Dunedin Multidisciplinary Health and Development Study, a prospective longitudinal study of a representative ($n = 1037$) birth cohort.[48,49] In terms of human development, the rat age span of 6–14 months corresponds roughly to 18–40 years,[50] with a shift to somewhat older human age windows in mice of the same age.[51]

Among the most common consequences of perinatal asphyxia in humans are cognitive abnormalities later in life, ranging from learning disabilities to developmental delay, mental retardation, and autism.[3–5,30] In children following perinatal asphyxia, impairments in memory were found to be associated with a reduced volume of the hippocampus, a brain region that is specifically vulnerable to asphyxia.[4] Similar to the clinical findings, marked cognitive impairment, as evidenced by deficits in learning and memory, was a later life consequence in the present model. Furthermore, neurodegeneration was observed in the dorsal hippocampus, which, together with the aberrant MFS in the CA3 region, explains the decline in spatial learning.[32,33,52] Although, to our knowledge, aberrant MFS has not yet been described as a later life consequence of BA in humans, it is a well-known structural alteration in patients with mesial temporal lobe epilepsy, which is the most common type of epilepsy in adults.[30]

The behavior of postasphyctic rats in the six-arm RAWM model of spatial learning and memory used here resembled impulsive behavior as observed in young rats (P30–P76) in an eight-arm RAWM paradigm following hypoxic–ischemic brain injury in P7 rats.[53] Impulsivity is a core symptom of attention-deficit/hyperactivity disorder—a diagnosis common in children with BA/HIE.[3,54] Following BA/HIE, impulsive behavior can occur along with memory impairment, most likely as a consequence of neurodegeneration in the hippocampus and striatum, which have been associated with specific cognitive functions such as memory and attention.[3,54] More specific tests of impulsivity, as previously used in hypoxia–ischemia and hypoxia-only rat models,[55–57] are needed to characterize in more detail the altered behavior of the postasphyxial rats in the RAWM observed here.

In addition to the morphological alterations in the hippocampus, neuronal loss was observed in the paraventricular and mediodorsal thalamus of the postasphyxial rats. Neuroimaging and neuropathological studies have revealed that the thalamus is among the selectively vulnerable brain regions following HIE in the human newborn and may contribute to sensorimotor deficits.[5,30,58] In human neonates with HIE, injury to the thalamus is typically not observed in isolation, but in association with damage to the hippocampus,[5,30] as also observed in the present animal model.

Epilepsy represents a common outcome of newborns with neonatal seizures, especially in those with severe brain injury and additional neurodevelopmental disabilities.[59] A high incidence of severe types of epilepsy has been described following HIE/neonatal seizures, including infantile spasms (West syndrome), early myoclonic encephalopathy, and early infantile epileptic encephalopathy, but the occurrence of such syndromes is strongly determined by the underlying etiology of HIE.[59] Other seizure types that have been described include focal and generalized seizures similar to those observed here, often coexisting in the same patient and poorly responding to therapy.[59–61] In the present study, 100% of the postasphyxial rats exhibited SRS, recorded ~10 months after asphyxia/neonatal seizures. Three types of SRS were observed: electrographic seizures without obvious clinical correlates, nonconvulsive electroclinical seizures with focal behavioral seizure signs, and, rarely, convulsive electroclinical seizures. The frequency of the SRS varied widely from a few seizures to hundreds of seizures per week. Electrographic and nonconvulsive electroclinical seizures occurred at low frequency also in sham controls, which has been reported in previous studies in other rodent models, including the hypoxia-only model of HIE in rats.[62] Paroxysmal EEG patterns have been observed in various rat strains.[29] Such patterns may, at least in part, result from structural or functional alterations caused by the invasive electrodes during chronic EEG recordings.[29,63,64]

Interestingly, in postasphyxial rats, we found significant correlations between the frequency of SRS and loss of neurons in the dentate hilus and the extent of aberrant MFS in the CA3. The correlation between seizure frequency and cellular effects begs the question of whether it was the asphyxia and/or neonatal seizures per se that initiated a very long maturational process that culminated in epilepsy; or whether there was a much earlier onset of spontaneous electrographic seizures that, over time, led to progressive cellular damage and to the behavioral changes. This is an important issue that should be addressed in further studies.

As described above, aberrant sprouting of mossy fibers is often a consequence of neuronal loss in the dentate hilus,[36] which is the most susceptible region of the hippocampal formation following different types of brain injury[35,65] but is relatively resistant to damage in hypoxia-alone and hypoxia–ischemia models of HIE.[14] However, data concerning damage to the hilus in hypoxia-alone and hypoxia–ischemia models of HIE are limited and not quantitative. Furthermore, the P7 rat (used in many HIE models) is less susceptible to hippocampal neurodegeneration in response to hypoxia/asphyxia and ischemia than the P10–P12 rat.[43] Moreover, specific populations of hilar neurons could be lost, and this would not be known unless appropriate staining procedures were combined with quantitative analyses to address this question. This prompted us to count mossy cells and different subtypes of GABAergic interneurons in the hilus, which indicated that mossy cells and parvalbumin-positive GABAergic interneurons were preferentially lost following asphyxia.

Hilar cell loss as well as increased and aberrant MFS are observed in cases of human temporal lobe epilepsy and many experimental models of epilepsy.[35] MFS has been associated with epileptogenesis in animal models, although a direct cause–effect relationship has long been a matter of debate.[35,36,65,66] More recent preclinical studies suggest that the new (synaptoporin-positive) neurites establish functional synaptic connections and may contribute to the state of hyperexcitability that either provokes or facilitates abnormal discharges.[35,67] In a hypoxia-only model of neonatal seizures, aberrant MFS was observed in the stratum oriens of the CA3 hippocampal region,[62,68] similar to the MFS in the present model. The altered mossy fiber distribution was a likely explanation of the enhanced synaptic activity seen at CA1:CA3 synapses following neonatal seizures[69] and the disturbance in cognitive function seen in later life.[70,71] Although in the present model and hypoxia-only models of HIE, MFS was restricted to the CA3 region, aberrant MFS into the molecular layer of the dentate gyrus has been observed in more severe models of HIE.[14]

We recently reported a loss of neurons in the CA1 region of the hippocampus shortly (24 h) after asphyxia, which seemed to be a consequence of neonatal seizure-induced apoptosis.[72] This is consistent with findings in other HIE models that the CA1 (and CA3) regions are particularly sensitive to hypoxia and ischemia.[73–75] In the present study, we found a significant loss of CA3c neurons in both dorsal and ventral hippocampus, but unexpectedly, we did not observe a corresponding fall in the number of CA1 neurons at 14 months after asphyxia. One possible explanation would be an adaptive change in the overall morphology of the hippocampus, with long-term rewiring of the hippocampal circuitry and rearrangement of CA1 neurons. This explanation is, however, difficult to verify or falsify.

In conclusion, there is increasing preclinical and clinical evidence that neonatal seizures increase the risk of adverse outcomes following HIE.[12] Thus, new treatments that more efficaciously suppress neonatal seizures and the later life consequences of HIE and neonatal seizures are urgently needed. The rat model of BA[17] employed in the present study provides a novel tool to develop such treatments. As shown here, this model recapitulates a number of salient later life clinical outcomes of BA and subsequent neonatal seizures, including damage to susceptible brain regions, cognitive decline, anxiety, and epilepsy. Results from animal research suggest that neonatal seizures may exacerbate HIE-induced brain injury and neurodevelopmental outcome,[9,76,77] whereas this is a matter of debate in humans.[2,9,11,12,59] Because seizures occur after asphyxia in the present model, it can be employed in future studies to examine the controversial and important question of whether antiseizure drugs that block the postasphyxia seizures[20] will decrease the incidence or severity of later life consequences.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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