Maternal surgery during pregnancy has a transient adverse effect on the developing fetal rabbit brain

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BACKGROUND: Recently, the US Food and Drug Administration called for cautious use of anesthetic drugs during pregnancy. In 0.2−2% of pregnancies, nonobstetric surgery is being performed. The consequences of anesthesia during pregnancy on fetal development remain unclear, and preclinical studies in relevant animal models may help to elucidate them.

OBJECTIVE: To assess the effect of maternal anesthesia and surgery during pregnancy on the developing fetal brain, using a rabbit model.

MATERIALS AND METHODS: This is a randomized, sham-controlled study in time-mated pregnant does at 28 days of gestation (term = 31 days), which corresponds to the end of the second trimester in humans. Anesthesia was induced in 14 does (155 pups) with propofol and maintained with 4 vol% (equivalent to 1 minimum alveolar concentration) sevoflurane for 2 hours, and a laparotomy with minimal organ manipulation was performed (surgery group). Maternal vital signs (blood pressure, heart rate, peripheral and cerebral oxygen saturation, temperature, end-tidal CO_2, pH, lactate) were continuously monitored. Sham controls consisted of 7 does (74 pups) undergoing invasive hemodynamic monitoring for 2 hours without sedation. At term, does underwent cesarean delivery under ketamine−medetomidine sedation and local anesthesia. Pups either underwent motor and sensory neurologic testing followed by euthanasia at day 1 or daily neurodevelopment testing for 2 weeks and extensive neurologic assessment at 5 and 7 weeks (open field and object recognition test, T-maze, and radial-arm maze). Brains were harvested for histologic assessment of neuron density and synaptophysin expression.

RESULTS: Blood gases and vital parameters were stable in both groups. On postnatal day 1, surgery pups had significant lower motor (25 ± 1 vs 23 ± 3; $P = .004$) and sensory (16 ± 2 vs 15 ± 2; $P = .005$) neurobehavioral scores and lower brain-to-body weight ratios (3.7% ± 0.6% vs 3.4% ± 0.6%; $P = .001$). This was accompanied by lower neuron density in multiple brain regions (eg, hippocampus 2617 ± 410 vs 2053 ± 492 neurons/mm^2; $P = .004$) with lower proliferation rates and less synaptophysin expression. Furthermore, surgery pups had delayed motor development during the first week of life, for example with hopping appearing later (6 ± 5 vs 12 ± 3 days; $P = .011$). Yet, by 7 weeks of age, neurobehavioral impairment was limited to a reduced digging behavior, and no differences in neuron density or synaptophysin expression were seen.

CONCLUSION: In rabbits, 2 hours of maternal general anesthesia and laparotomy, with minimal organ and no fetal manipulation, had a measurable impact on neonatal neurologic function and brain morphology. Pups had a slower motoric neurodevelopment, but by 7 weeks the effect became almost undetectable.

Key words: brain, cognition, development, fetus, general anesthesia, long term, maternal surgery, motor, neurobehavior, pregnancy, rabbit

Annually, 0.2−2% of pregnant women in Europe and the United States undergo surgery during gestation.[1−6] This might be for nonobstetric indications, for example, appendicitis, cholecystitis, or, more rarely, for fetal surgery, often performed under general anesthesia (GA). In the past decade, there has been growing concern over the potential detrimental effect of anesthesia on the developing brain. Clinical studies have found conflicting results, either showing an altered neurodevelopmental outcome, abnormal behavior, and learning difficulties or showing no difference at all.[7] Mainly during the brain growth spurt, the developing brain appears to be extremely vulnerable to external factors, including anesthetic drugs.[8] The brain growth spurt is the period during which the brain is at its peak growth, when synaptogenesis takes place. In humans, this phase starts at mid-gestation and continues through the first years of life. This means that the majority of nonobstetric (second trimester) and fetal procedures under general anesthesia (end of second trimester) are performed while the brain is most sensitive.[9,10] Suspected anesthetics are γ-aminobutyric acid (GABA) receptor agonists (eg, isoflurane, sevoflurane, benzodiazepines, propofol, and others) and *N*-methyl D-aspartate (NMDA) antagonists (ketamine).

Multiple animal studies investigating the effects of anesthetic drugs in the neonatal period show increased neuronal apoptosis and decreased synapse formation as well as learning impairment and lower memory function.[11,12] In pregnancy, most of these drugs will freely cross the placenta and lead to similar outcomes.[13−15] Until now, these studies exposed fetuses to maternal anesthesia only, whereas clinically, general anesthesia is administered to pregnant women almost only for a surgical purpose. The additional effect of surgery has not been well studied. Study is required, as surgery induces an inflammatory process that in turn may interfere with brain development.[16,17]

In this study, we aimed to investigate the combined effect of standardized maternal surgery under GA at the onset of the fetal brain growth spurt in a rabbit model, which corresponds to the end of the second trimester in humans. We hypothesized that maternal anesthesia combined with surgery would lead to a

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AJOG at a Glance

Why was the study conducted?

– Human data on the fetal impact of maternal surgery in pregnant women are lacking.

– Preclinical data on the impact of single and short exposure to anesthesia during pregnancy on fetal brain development are conflicting.

– Preclinical data on surgery during pregnancy are lacking.

Key findings

– Surgery during pregnancy leads to lower neuron and synaptic density, and consequently to motor and sensory deficits at birth.

– Animals develop more slowly.

– These differences fade over time.

What does this add to what is known?

– This study demonstrates short- and long-term effects of maternal surgery during pregnancy.

– This study shows how the impact evolves over time.

poorer neurologic outcome with histologic changes at birth, and that this would lead to behavioral and cognitive impairment in the long term.

Material and Methods

Time-mated rabbits (hybrid of Dendermonde and New Zealand White rabbit) were housed in individual cages at 21°C, 42% humidity, with a 12-hour day–night cycle and free access to water and food. Animals were treated according to current guidelines for animal well-being, and experiments were approved by the Ethics Committee for Animal Experimentation of the Faculty of Medicine (P078/2017). Pregnant does were randomized to undergo a standardized laparotomy under GA (n = 14; further referred to as "surgery") or sham manipulation without sedation (n = 7; "sham").

Anesthetic and surgical procedures

At gestational day, 28 does were placed in a transparent induction chamber (to observe respiration rates) filled with 8% sevoflurane and 80% oxygen for 5 minutes, then placed on the operating table while maintaining sevoflurane anesthesia with a mask. Intravenous and arterial catheters were placed in the ear and an intravenous (IV) bolus of propofol 6.5 mg/kg (Propovet, Zoetis Belgium, Oostkamp, Belgium), and fentanyl 5 μg/kg (Fentanyl, Janssen-Cilag NV, Beerse, Belgium) was given thereafter. The animal was endotracheally intubated and placed in the left lateral decubitus position. Normoventilation was obtained with an inspiratory oxygen concentration of 30%, using a closed-circuit respirator (FelixDualTM; Air-Liquide Medical Systems). Sevoflurane was continued throughout the procedure at 1 minimum alveolar concentration (MAC) (3.8 vol%) under continuous monitoring, as detailed in Supplement 1.

A median laparotomy (5 cm) partially exposing the uterus allowed micro-ultrasound Doppler (Vevo 2100 Imaging System, VisualSonics Inc, Toronto, ON, Canada) evaluation of the fetal heart rate in 2–4 fetuses without additional manipulation. At the end of surgery, enrofloxacine (10 mg/kg IV) (Baytril 5%, Bayer, Diegem, Belgium), medroxyprogesterone acetate (9 mg/kg, subcutaneously [SC]) (Depo-Provera, Upjohn, Puurs, Belgium), and lidocaine 4 mg/kg (wound infiltration) (Xylocaine, AstraZeneca, Brussel, Belgium) were administered. Animals were awakened after exactly 120 minutes of anesthesia and allowed to recover in their cages. Sham animals were partially restricted in a cage (59 × 41 × 37cm). Under local anesthesia, IV and intraarterial lines were placed and the animals were monitored for 120 minutes (Supplement 1). A blood sample for gas analysis was taken after intubation (baseline) and before awakening (end of the experiment). In the sham group, this was taken in the beginning and at the end of blood pressure monitoring. Rabbits were monitored daily using the Rabbit Grimace Scale[18] (Supplement 1).

Delivery

Cesarean delivery on day 31 (term) was performed in both groups (sham and surgery) under ketamine/medetomidine sedation (ketamine, 15 mg/kg intramuscularly [IM]; Nimatek; Eurovet Animal Health BV, Bladel, The Netherlands; and medetomidine, 25 mg/kg IM, Domitor, Orion Pharma, Aartselaar, Belgium) and local anesthesia infiltration with lidocaine 4 mg/kg (Supplement 1). Time between falling asleep and delivery was kept to less than 5 minutes. Pups were kept in an incubator and gavage fed. On day 1 pups were randomized to either a short (1-day) or

TABLE 1
Blood gas values at the beginning and end of the procedure

	Sham (start)	Surgery (start)	Sham (end)	Surgery (end)
pH	7.45 ± 0.02	7.47 ± 0.07	7.41 ± 0.03	7.43 ± 0.05
pCO_2 (mm Hg)	28.0 ± 4.0	31.2 ± 4.7	30.4 ± 4.6	34.5 ± 4.9
pO_2 (mm Hg)	85.9 ± 8.1	169.5 ± 57.2	88.1 ± 7.4	138.8 ± 24.8
Lactate (mm Hg)	4.2 ± 2.7	3.7 ± 1.3	1.1 ± 0.4	4.4 ± 2.1

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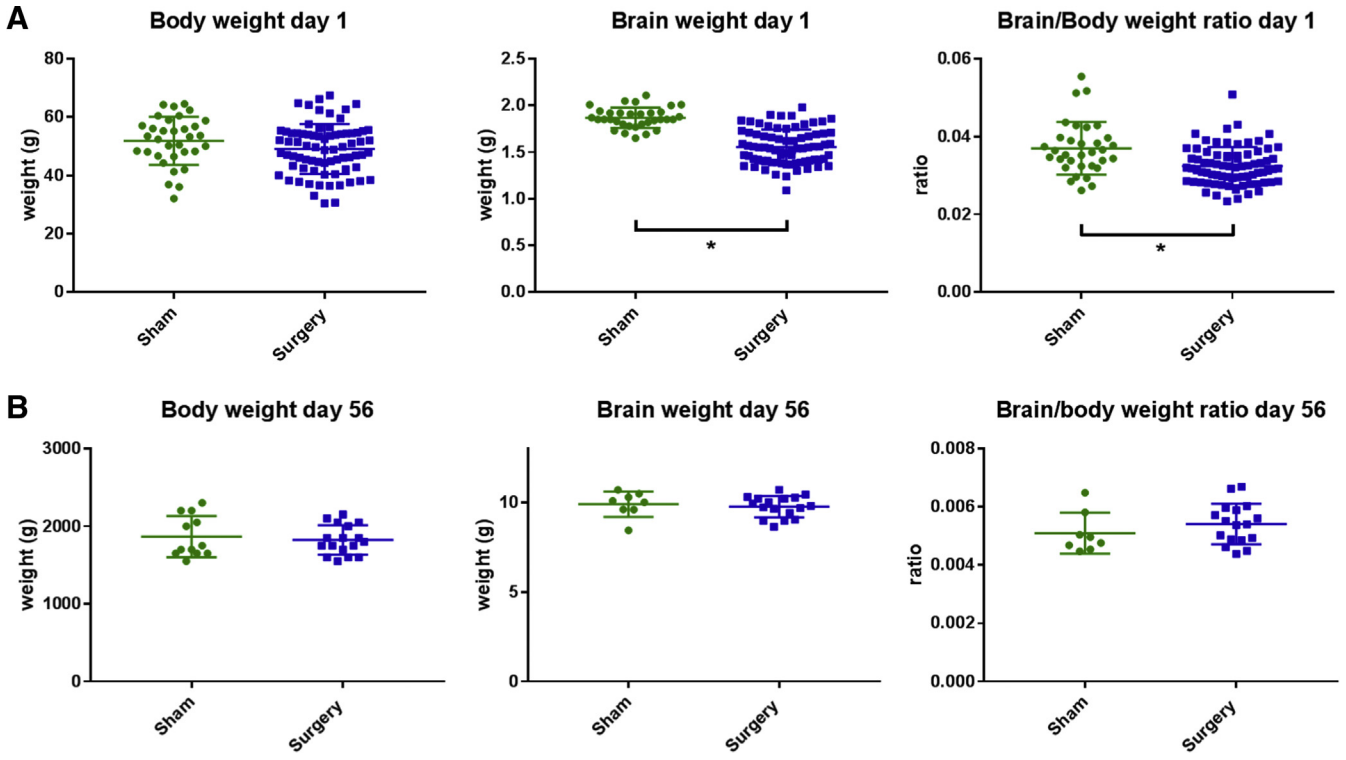
FIGURE 1
Biometrics: body weight, brain weight and brain-to-body weight ratio at A, Postnatal day (PND) 1, and B, PND 56. Data are presented as individual data points, with mean and standard deviation. *$P < .05$



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long-term (56-day) group. The latter group was raised by cross fostering from day 1 (Supplement 2).

Neurobehavioral assessments

Short-term motor assessment comprised scoring of gait, posture, locomotion, activity, and tone. Afterward, the cranial nerves, pain response, and righting reflex were tested for sensory evaluation as described previously.[19] In the long-term group, a behavioral maturation score was assessed daily on days 1−14, and 11 aspects of the rabbits' physical and neurobehavioral development were recorded. Thereafter, on days 32−36 and 46−56, an open field test (OFT), T-maze, novel object recognition task (NORT), and radial-arm maze assessment were performed. All assessments were filmed and scored by an observer blinded to the treatment groups. A full description of assessments can be found in Supplement 3.

Neuropathological assessments

On day 1 or day 56, animals were transcardially perfusion fixated after ketamine/xylazine sedation (ketamine (35 mg/kg IM) and xylazine (6 mg/kg IM), XYL-M; VMD, Arendonk, Belgium). Brains were extracted, paraffin embedded, and serially sectioned at 4 μm every 50 μm as described previously.[19] Sections were stained with Cresyl Violet (C5042-10G; Sigma-Aldrich, Overijse, Belgium), mouse monoclonal anti-human Ki67 (M724001-2; Agilent, Diegem, Belgium), mouse monoclonal anti-synaptophysin (Sy38, ab8049; Abcam, Cambridge, UK), and a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method for fluorescent in situ end labeling of double-stranded DNA fragmentation (Apoptag S7110; Millipore, Billerica, MA). Four relevant brain areas were evaluated, namely, the prefrontal cortex, caudate nucleus, hippocampus, and thalamus. Further details and quantification methods can be found in Supplement 4.

Statistical analysis

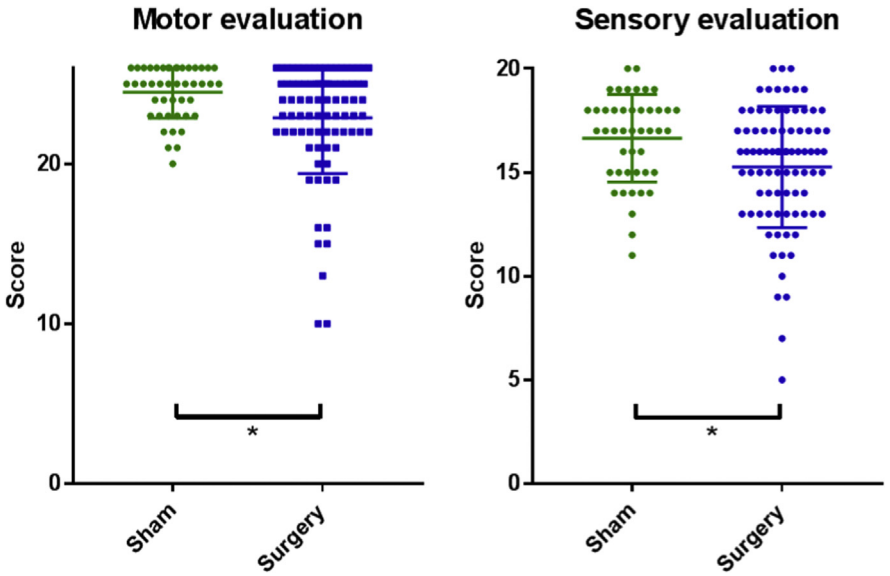
Data were analyzed using SPSS version 24 (IBM Corp, Armonk, NY). Data distribution was checked for normality using a D'Agostino−Pearson omnibus normality test. Comparison was done by an unpaired Student t test or Mann−Whitney test. A P value of $<.05$ was considered significant. Blood pressure and heart rate were compared using a 2-way analysis of variance. All data are presented as mean with standard deviation.

Results

Maternal and neonatal outcomes

Rabbits undergoing surgery (n = 14) remained stable throughout the whole procedure; in addition, no fetal bradycardia or fetal death was noted (Supplemental Figure1). Compared to the sham group (n = 7), surgery rabbits had a higher pO_2 (85.9 ± 8.1 vs 169.5 ±

FIGURE 2
Motor and sensory neurobehavioral outcome on postnatal day (PND) 1. Data are presented as individual data points, with mean and SD. *$P < .05$



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57.2 mm Hg; $P = .001$ beginning, 88.1 ± 7.4 vs 138.8 ± 24.8 mm Hg end; $P < 0.001$), whereas sham rabbits displayed a drop in lactate levels by the end of the procedure (3.7 ± 1.3 vs 1.1 ± 0.4 mmol/L; $P = .04$) (Table 1). Heart rates were similar, and blood pressure differed only at 10 and 15 minutes after the start of the procedure (Supplemental Figure 2). There was no hypertensive response to intubation.

At delivery, litter sizes (10.6 ± 4.2 vs 11.1 ± 3.4; $P = 1$) were comparable, but fetal survival rates were higher in the surgery group (69/72, 93.5% ± 8.9% sham vs 102/155, 65.6% ± 38.3% surgery; $P = .0001$). Following randomization, 41 sham and 83 surgery pups were assigned to the short-term study, and 16 sham and 19 surgery pups were assigned to long-term study.

Short-term outcome

Surgery pups had smaller brains (1.9 ± 0.1 vs 1.6 ± 0.2 g; $P < .001$) but comparable birthweight (51.4 ± 7.8 vs 48.7 ± 8.3 g, $P = .07$), resulting in a lower brain-to-body weight ratio (3.7% sham vs 3.2%; $P = .001$) (Figure 1A).

Upon neurobehavioral assessment, surgery pups had a significant motor deficit (mean motor score of 24.5±1.6 sham vs 22.9 ± 3.5; $P = .007$) (Figure 2 and Video 1). This was attributable mainly to a lower gait ($P = .01$), locomotion ($P = .02$), and motor activity of the head ($P = .047$), hind limbs ($P = .03$), and front limbs ($P = .02$). In addition, lower sensory scores (16.6 ± 2.1 sham vs 15.3 ± 2.9; $P = .01$) due to the inability to suck and swallow ($P = .02$) were seen in the surgery pups.

Neuropathological assessment showed lower neuron densities in the prefrontal cortex (−16%), caudate nucleus (−15%), and hippocampal dentate gyrus (−22%) in the surgery pups (Figure 3A). The mean integrated density of synaptophysin was up to 34% lower in the surgery group. (Figure 4A). These differences were accompanied by a lower percentage of Ki67-positive cells in the surgery group (Supplemental Figure 3). No differences were seen in the expression of apoptotic and Iba-1−positive cells (Supplemental Figures 4 and 5).

Long-term outcome

On neurodevelopmental assessment during the first 2 weeks of life, surgery pups displayed delayed motor development, ascribed to later appearance of spontaneous hopping, prolonged dragging of the hind limbs, and less forward movement and displacement (Figure 5).

At postnatal day (PND) 32, surgery rabbits displayed a higher rate of spontaneous alteration in the T-maze test (81.9 ± 27.9 vs 45.1 ± 36.7 %; $P = .012$). In addition, surgery rabbits displayed a lower exploration time in the NORT (54.9 ± 36.2 seconds vs 23.1±20.0 seconds; $P = .005$). However, no behavioral differences were noted between the 2 groups in the OFT (Table 2).

At PND 46, surgery rabbits displayed less active digging behavior in the OFT (2/10 vs 6/7; $P = .015$). No other differences were noted in the OFT, NORT, or T-maze tests (Table 2).

At PND 53 and 56, a total of 4 sham and 5 surgery rabbits underwent radial-arm maze testing. None of these rabbits consumed enough bait (apple), so no learning curve could be determined and there was no further analysis of this parameter.

On PND 56, body weights (1867 ± 265 vs 1823 ± 190 g; $P = ,6$) and brain-to-body weight ratios (0.51±0.07 vs 0.54 ± 0.7; $P = .3$) were not significantly different (Figure 1B). Overall mortality was comparable (4/16 vs 2/19; $P = .3$). No clear etiology for postnatal death could be determined for these cases. Upon neuropathological assessment, no significant differences were noted in neuron densities and synaptophysin staining (Figures 3B and 4B).

Comment

We studied the effects of a standardized laparotomy under GA on the developing fetal rabbit brain at a timepoint correlating to human mid-gestation. Compared to control equivalents, exposed pups displayed significant neonatal and early postnatal neurodevelopmental impairment. However, by preadolescence, these differences became almost unrecognizable.

There is growing preclinical evidence that GA has adverse effects on the developing brain.[8,13,14,20−31] We chose to investigate such effects in a rabbit model, because this species develops its brain mainly in the perinatal period, like humans, and unlike rodents (postnatal),

sheep, or nonhuman primates (prenatal). Aiming to simulate a clinical scenario, we studied the effects of a combination of surgery and anesthesia. We anesthetized does at day 28, which is at the beginning of the brain growth spurt,[32] corresponding to the human end of the second trimester, the period in which most surgery is performed in pregnant women. Surgery induces tissue injury and an inflammatory response (release of inflammatory cytokines, eg, interleukin-6, tumor necrosis factor−α, interleukin-1β), which may also adversely affect brain development.[33,34] In this experiment, we compared surgery under anesthesia with a sham procedure. In the sham group, we restrained the animals, which apparently induced a transient increase in heart rate and blood pressure, which, however, fell after into the normal range after 10 minutes (Supplemental Figure 2). This may correspond with the initial stress caused by restraining the animals in the surgery group to undergo anesthesia.

During the experiments, the cardiopulmonary status of the does remained stable, as evidenced by arterial pressures within the physiological range, maintained cerebral oxygen saturation, and stable oxygen saturation. In addition, fetal hemodynamics most likely remained stable, although the measurement of fetal heart rate twice during the experiment is certainly only a very basic form of fetal monitoring.

We measured prominent effects of anesthesia and surgery in early postnatal life at different levels. Exposed pups had lower brain weights and volumes but normal body weights. These gross anatomical findings coincided with reduced proliferation, as earlier demonstrated in rodents.[35−39] The observed reduction in proliferation taking place over the last 3 days of gestation (~10% of pregnancy duration), coincided with a 9% decrease in brain/body weight ratio. We measured lower neuron densities in the prefrontal cortex, caudate nucleus, and hippocampal dentate gyrus, which are functionally relevant areas in humans and animals.[12,40] This observation is in line with findings in other species.[8,13,20−25,27] These studies revealed that lower neuron densities coincide with increased apoptosis within 1 day after the insult. Several underlying mechanisms were suggested, including direct neuronal excitation[41,42] and exposure to pro-inflammatory cytokines.[43−46] In our study, we did not examine apoptosis or neurodensity immediately after the insult, because we aimed primarily for functional outcomes rather than mechanisms. Brains were investigated in the neonatal period, 4 days after the combined insult, when differences in apoptosis may no longer be measurable. Therefore, although a profound elevation of apoptosis is not expected at this time period, these processes may have taken place. Apoptosis

FIGURE 3

Neuron density. A, Representative image of the dentate gyrus in a sham (left) and surgery (right) animal stained with Cresyl Violet. B, Neuron density in the 4 regions of interest on postnatal day (PND) 1. C, Neuron density in the 4 regions of interest on PND 56. Data are presented as individual data points, with mean and standard deviation. *$P < .05$

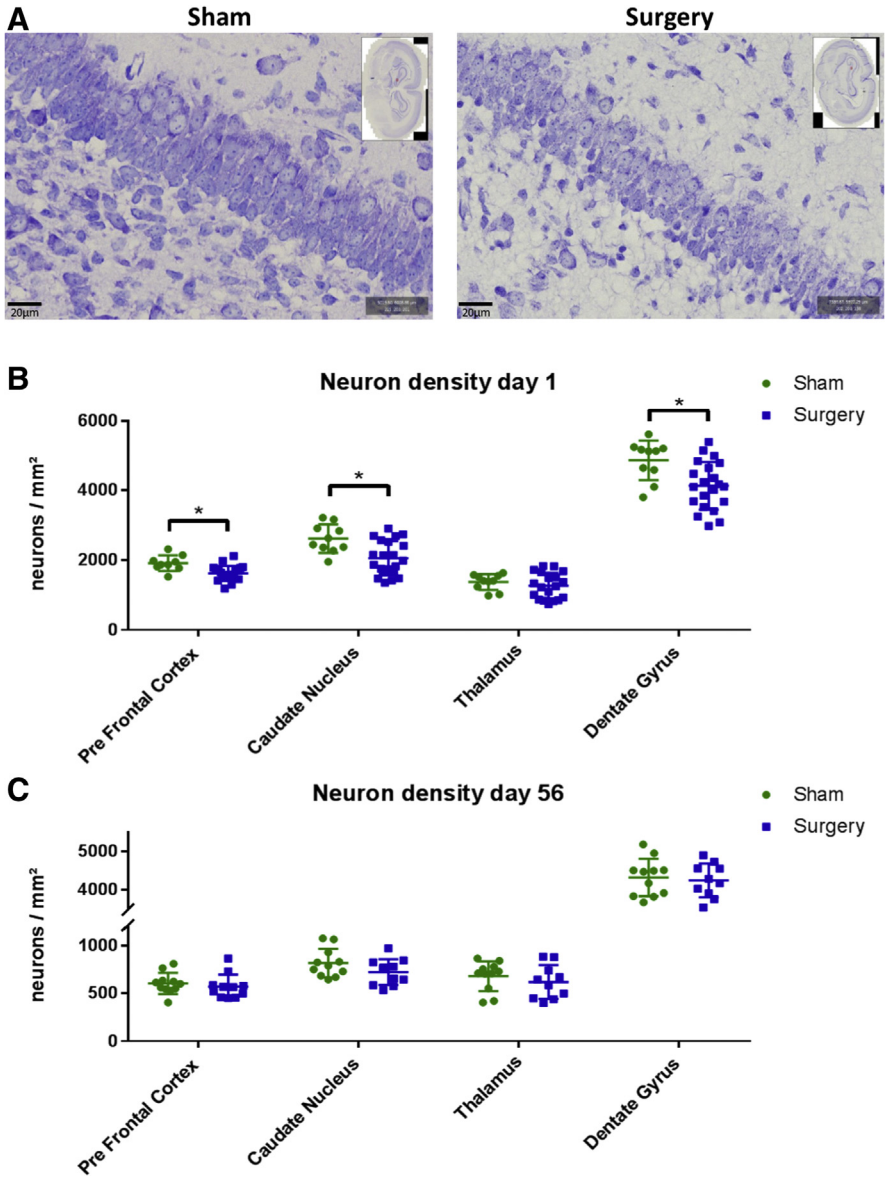

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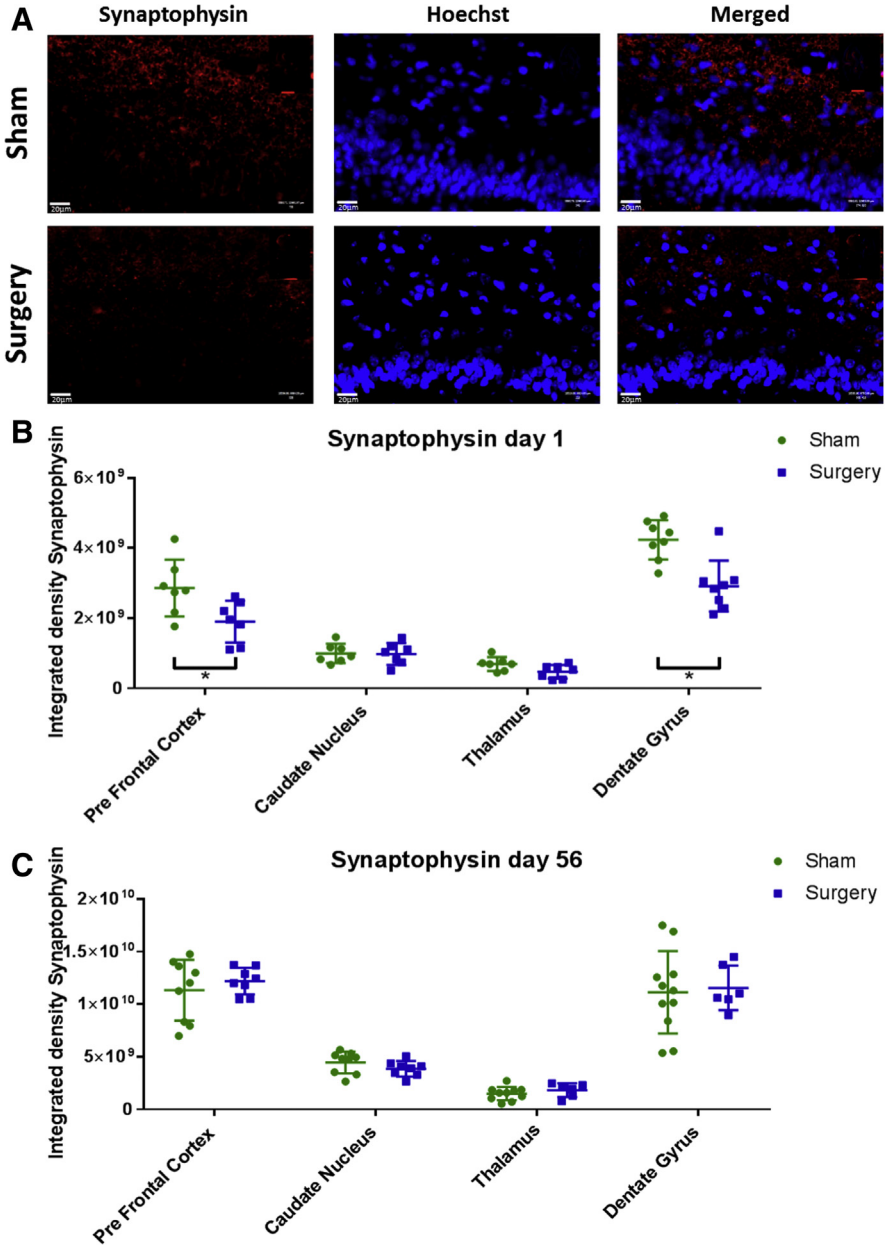
FIGURE 4
Synaptic density. A, Representative image of the dentate gyrus in a sham (top) and surgery (below) animal stained with synaptophysin. B, Integrated density synaptophysin in the 4 regions of interest on PND 1. C, Integrated density synaptophysin in the 4 regions of interest on postnatal day (PND) 56. Data are presented as individual data points, with mean and standard deviation. *$P < .05$



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has been shown to be no longer demonstrable as early as 48 hours after exposure to dizocilpine.[21] Dizocilpine is a noncompetitive NMDA antagonist,[47] which is presumed to impact the fetal brain through a similar mechanism as sevoflurane.[48] The presence of in utero deceased fetuses, which is not uncommon in rabbits, may also have had an effect on neurodevelopment, for example, by the release of inflammatory agents.[16,17] To assess such an effect, we repeated the statistical analyses by excluding the 5 does in which more than half of the fetuses died; however, we found nearly the same differences between anesthesia-exposed and control fetuses. We also performed mixed model analysis to cluster pups per mother. This lowered the significance of the differences but led to the same conclusion.

We also observed lower synaptic density in the prefrontal cortex and the hippocampus. Anesthetics are known to interfere with synaptogenesis through binding with neurotransmitter receptors,[49,50] which play a primary role in synapse activation and strengthening.[51–53] In this study we used synaptophysin, because this membrane protein is a marker for presynaptic vesicles, which are present in almost all neurons. In further research, it might be interesting to examine the effects of anesthesia in specific synapse types, such as inbibitory or excitatory, their location (axoaxonic, axodendritic, or axosomatic), or the nature of the neurotransmitters involved.

Mechanisms other than anesthetic-induced neurotoxicity may certainly play a role, including the release of inflammatory cytokines, which also interfere with synaptogenesis and synaptic plasticity.[54,55] In this experiment, we used limited indirect measures of inflammation; yet, like others, we could not show a difference in gliosis.[56]

This experiment also involved progressive sensory (day 1), motor (day 1 up to day 14) as well as neurocognitive (days 32–36 and days 46–56) function testing. The most striking observation was that initial functional differences became smaller over time. In those animals that were followed-up longitudinally, the effects were fading, with only 1 persistent difference: namely, digging behavior (46 days), coinciding with comparable histology in both groups. Digging is a pattern of mature behavior that rarely occurred at PND 32. The progressive decrease in impact contrasts with findings in rodents, in which, except for 1 study in pregnant mice,[57] long-term neurocognitive dysfunction following anesthesia has been consistently demonstrated.[14,29,38,58–60] We can

only speculate about these apparently contradictory findings. This may be purely species specific. It might be that rabbit pups are initially impaired but do indeed recover. Functional recovery is usually explained through brain plasticity, which is the ability of the central nervous system to adapt in order to mature or in response to changes in the environment or lesions.[61,62] Brain plasticity is essential and physiologic for species that are immature at birth, such as humans and rabbits.[63] Notably, in terms of plasticity, the rodent and rabbit brain differ substantially.[64] Appropriate social housing and interactions, which we stimulated through the housing circumstances, have been shown to stimulate the expression of social and species-specific behaviors such as exploration.[65,66] Furthermore, Zhang et al found a similar transient effect on cognition in humans.[67]

In this study, the interval between insult and delivery was very short (3 days). In rabbits, developmental stages of different organs are rushed through at a pace not comparable to that in man. The experiment was carried out at a timepoint when the brain is rapidly developing and the transitions of neurogenesis and synaptogenesis are abundant. It remains unclear why, in this model, rabbits seemed to "recover" from the neurobehavioral impairment. We speculate that this may be due to neuroplasticity, a process that was observed earlier in rabbits with intrauterine growth restriction.[68] In the latter, the insult is chronic and fetuses are born with a lower birth weight, which may make them more vulnerable and may permit only partial improvement. All this remains speculative and has to be further studied in experiments designed for the purpose of elucidating this. Conversely, it may well be that the neurocognitive tests or morphometric scores used in our study were not sensitive enough to detect more subtle sustained impairment. In fact, 2 months after the combined insult, some subtle (ie, digging) differences persisted.

We acknowledge several limitations to our study design and model. First, the duration of anesthesia compared to the total pregnancy duration is obviously disproportionally longer than in humans. Second, although the rabbit may have a developing brain similar to that of humans, it is far from equal. Third, functional testing was done immediately after birth. This is possible in rabbits, because accelerated maturation of motor abilities makes testing possible unlike in humans, which limits the translation of our findings.[69] Rabbits are also a species in which mechanistic research is hampered by the lack of molecular and genetic tools. We also acknowledge some flaws in the design of our study. First, we should, in retrospect, have included a group with anesthesia only, or even a group with a simulated maternal surgical procedure, so that we could have better disentangled the effects of anesthesia from those of anesthesia plus surgery.

Our study and model, however, also have some strengths. First, the rabbit is a perinatal brain developer, comparable to the human brain, with the brain growth spurt starting late in pregnancy (at day 28 of a 31-day gestation) and continuing after birth. This is in contrast to rats and mice (postnatal) or sheep and nonhuman primates (prenatal),[32] which is relevant to the current research question.[32] The rabbit has frequently been used to study the effect of perinatal insults, including prematurity,[19] intraventricular hemorrhage,[70,71] hypoxia,[69] and infection,[16] on neurodevelopment. Despite being relatively immature at birth, rabbits show a large repertoire of spontaneous behaviors (motor activities and reflex responses) that are well characterized[72–75] and can be reproducibly tested. Furthermore, rabbits have a placental structure (hemodichorial, ie containing 2 trophoblast layers) that is closer to that of humans (hemomonochorial, ie, containing 1 trophoblast layer) than that of rodents (hemotrichorial, ie containing 3

FIGURE 5

Neurodevelopmental patterns during the first 2 weeks of life. Hopping and dragging are presented as a Kaplan–Meier survival curve with the day on which a rabbit pup acquired a mature behavior, that is, started hopping in more than 50% of steps or stopped dragging its hind legs. Daily activity levels are expressed as the number of grid lines crossed in the open field in 90 seconds. Data are presented as individual data points, with mean and standard deviation. *$P < 0.05$

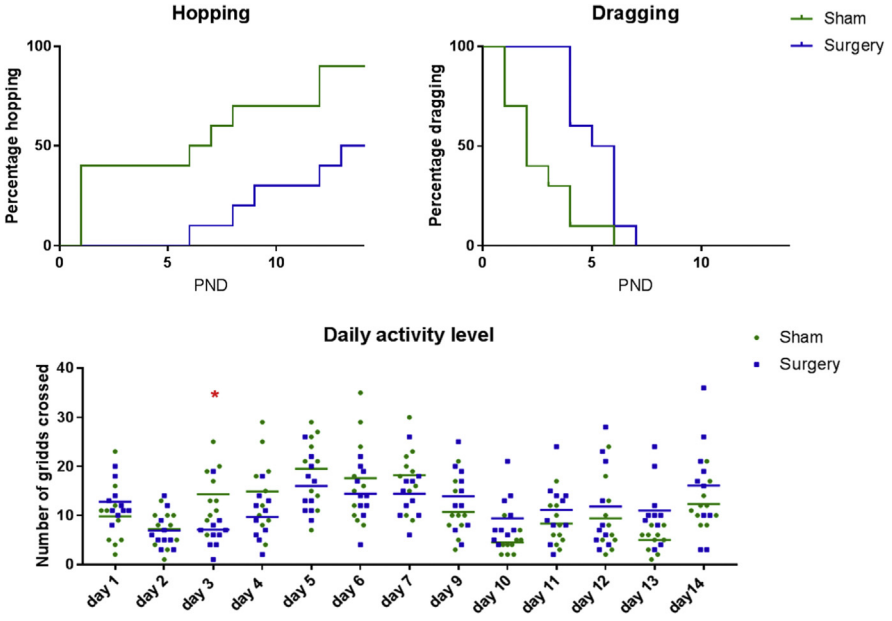

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TABLE 2
Outcome of neurobehavioral testing at 5 and 7 weeks

	Week 5			Week 7		
	Sham (n = 12)	Surgery (n = 17)	*P* value	Sham (n = 7)	Surgery (n = 10)	*P* value
Open Field Test						
Total Distance (m)	11.9 ± 0.5	12.4 ± 0.7	0.8	16.4 ± 0.9	14.8 ± 0.7	0.9
Time in starting area (s)	10.8 ± 7.7	8.4 ± 5.8	0.4	10.4 ± 8.6	4.8 ± 2.8	0.2
Time in central area (s)	22.1 ± 21.7	41.5 ± 47.8	0.3	45.9 ± 19.4	28.5 ± 28.9	0.6
Time in corners (s)	0.2 ± 0.6	1.8 ± 7.5	0.9	5.3 ± 13.1	32.2 ± 94.2	0.7
Time self-grooming (s)	17.0 ± 11.7	28.7 ± 24.8	0.2	40.6 ± 41.0	110.1 ± 142.3	0.2
Escapes attempts (n)	5.0 ± 4.4	3.0 ± 2.7	0.3	9.4 ± 5.8	10.9 ± 6.5	0.5
Times rearing (n)	4.7 ± 3.7	6.3 ± 5.9	0.7	5.5 ± 4.8	4.1 ± 3.9	0.7
Biting (yes/no) (%)	0.0	29.4	0.06	71.4	30.0	0.2
Digging (yes/no) (%)	25.0	11.8	0.6	85.7	20.0	0.015[a]
Urinating (yes/no) (%)	8.3	17.6	0.6	28.6	10.0	0.5
Defecating (yes/no) (%)	16.7	29.4	0.7	28.6	50.0	0.6
Novel Object Recognition Test						
Exploration in sample run (s)	54.9 ± 36.2	23.1 ± 20.0	0.005[a]	84.4 ± 64.4	111.2 ± 120.6	0.6
Novel object recognition ratio	0.29 ± 0.45	−0.08 ± 0.47	0.054	0.19 ± 0.58	0.35 ± 0.67	0.7
T-maze test						
Time in starting area (s)	12.1 ± 7.8	13.7 ± 9.7	0.9	19.8 ± 16.6	14.5 ± 7.5	1
Failure to choose (%)	11.1 ± 20.5	24.5 ± 39.1	0.6	9.5 ± 16.3	6.7 ± 21.1	0.6
Spontaneous alternation (%)	81.9 ± 27.9	45.1 ± 36.7	0.012[a]	47.6 ± 37.8	36.7 ± 29	0.7

[a] Statistically significant.

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trophoblast layers).[76] This is relevant for the passage of drugs.[77] Moreover, it is also considered suitable for long-term studies of perinatal insults[65,68,78,79] through a foster care system.[78] The pups can thus be followed longitudinally far beyond the neonatal age.

In conclusion, term pups, delivered from does exposed to 2 hours of GA for a maternal laparotomy at 28 days of gestation, had lower motor and sensory scores than unexposed controls. Exposed pups had smaller and lighter brains, with lower proliferation rates, and lower neuron and synaptic densities in multiple brain regions. These pups displayed slower neurological development in the first 2 weeks of life, and poorer cognitive function and altered species-specific behavior at 5 weeks. However, at 7 weeks of age, neurologic function tests could no longer discriminate surgery from sham rabbits, except that animals from mothers who had undergone operation displayed an abnormal digging behavior. ■

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Supplement 1: Maternal Manipulations

Once out of the induction chamber, animals were placed on a heating pad in left lateral tilt and gas anesthesia was continued with a mask. A saturation meter (M8048A, Philips Medizin System, Boeblingen, Germany) was applied on 1 ear and an arterial and venous line were placed in the opposite ear, connected to a NaCl 0,9% maintenance infusion (10 mL/kg/h). Intubation was performed under endoscopic guidance (11540AA and 11540KE, 1Karl Storz-Endoskope, Tuttlingen, Germany) with a 3.5-4.5 Ch polyethylene tube. Exhaled gases (oxygen, carbondioxide, sevoflurane) were continuously monitored (Felix Dual, Air Liquide Medical Systems, France). Temperature was measured orally, and near-infrared spectroscopy (NIRS) reading was installed on the back of the head (FORE-SIGHT MC-2030C Cerebral Oximeter, Casmed, Cas Medical Systems Inc, Branford, CT).[80] Sevoflurane was continued during surgery at 1 MAC (3.8%). Micro-ultrasound (Vevo 2100 Imaging System, VisualSonics Inc, Toronto, ON, Canada) examination was done through maternal laparotomy. Fetal heart rate was measured by Doppler ultrasound at 30 and 90 minutes after the start of anesthesia.

After closure of the skin, the incision site was injected with 4 mg/kg lidocaine (Xylocaine, AstraZeneca, Brussel, Belgium) and covered with aluminum spray. Enrofloxacine 10 mg/kg (Baytril 5%, Bayer, Diegem, Belgium) and medroxyprogesterone acetate 9 mg/kg (Depo-Provera, Upjohn, Puurs, Belgium) were administered prophylactically. The saturation meter was kept in place to allow continued monitoring until extubation, which was at the moment that spontaneous respiration returned.

Animals assigned to the sham manipulation group were placed in a 59 × 41 × 37-cm half-open non-transparent box. One ear of the rabbit was anesthetized locally using lidocaine 2% gel (Xylocaine, AstraZeneca, Brussels, Belgium). After 5 minutes, the rabbit was gently restrained, and venous and arterial lines were inserted as above. Rabbits were dressed in a baby bodysuit under which the lines were hidden to prevent biting and displacement. At the end of the manipulation, they were given enrofloxacine and medroxyprogesterone acetate as above.

In all does, blood gases were taken at baseline and at exactly 2 hours; other parameters were continuously monitored.

During the remainder of the pregnancy, does were monitored daily using the Rabbit Grimace Scale.[18] Using this scale, rabbits are observed for a couple of minutes and 5 action units are scored: orbital tightening, cheek flattening, nostril shape, whisker change and position, and ear shape and position. Each item is scored as 0 (not present), 1 (moderately present), or 2 (obviously present).

Cesarean delivery was performed at postconceptional day 31. After premedicating with intramuscular ketamine (15 mg/kg ketamine, Nimatek; Eurovet Animal Health BV, Bladel, The Netherlands) and medetomidine (25 mg/kg, Domintor; Orion Pharma, Aartselaar, Belgium), the doe was placed in the supine position, and local 2% lidocaine anesthesia was injected to allow medial laparotomy. The bicornuate uterus was exposed, and all pups were extracted through hysterotomy. Following delivery, the doe was euthanized with a mixture of 200 mg embutramide, 50 mg mebezonium, and 5 mg tetracain hydrochloride (intravenous bolus of 1 mLT61; Intervet International BV, Boxmeer, The Netherlands).

Supplement 2: Manipulation of Pups

After birth, pups were dried, stimulated, and kept in an incubator (TLC-50 Advance, Brinsea Products, Weston Super Mare, UK) set on 32°C and 55% humidity. Litters were separately housed in plastic boxes (20 × 25 cm). Pups were gavage fed twice daily with a Milk Replacer (FoxValley 30/50, Lakemoor, IL) supplemented with probiotics and colostrum (FoxValley 30/50, Lakemoor, IL) at 150 mL/kg feeding. At each feeding, their bladder was emptied by genital stimulation. On postnatal day (PND) 1, pups were randomized to either short- or long-term evaluation and marked by a permanent marker (short-term evaluation) or by implanting a subcutaneous microchip in the scruff for long-term follow-up (Compact Max Datamars, AgnTho's AB, Lidingö, Sweden).

The pups randomized to long-term evaluation were raised by a wet-nurse.[79] A doe that did not undergo any kind of manipulation was housed in a double cage to which a nesting box (54 × 31 × 27 cm) was connected. The foster mother raised a mixed group of 8−10 anesthetized and sham pups for a period of 4 weeks until they were fully weaned after, which the mother was euthanized.

Supplement 3: Neurobehavioral Testing

Before neurobehavioral testing was done, the animals' health was assessed. This included checking the animals daily in their housing and weighing them at least once per week. All videos were recorded and scored a posterior by a blinded observer.

PND1 evaluation

On PND1, all pups underwent neurobehavioral assessment, according to a protocol described by Derrick et al. (2004), which we used previously.[19,70] All the tests were performed out of the incubator and individually for each pup.

For neuromotor evaluation, an open field test was performed on a designated 30 × 30-cm area divided into quadrants over a period of 90 seconds. Each animal was left free to ambulate. Posture, gait, locomotion, motor activity of head, fore limbs and hind limbs, and duration of activity were evaluated and scored.

Locomotion was scored on the basis of the range of movement (ROM), the ability to walk and hop, and the distance traveled (/13).

Motor activity was assessed separately for head, fore limbs, and hind limbs, by grading the amount of spontaneous movement of each part (/9).

Limb tone was tested by passive flexion and extension of 1 of the hind limbs and graded according to a modified Ashworth Scale (/4).

The sum of these scores represented the total grade for this section, which was calculated out of a maximum of 26 points.

For neurosensory evaluation, senses were tested separately: soft sensation, sucking and swallowing, head turning during feeding, surface righting reflex, odor aversion to ethanol, and pain sensation.

Soft sensation was tested by gently rubbing the pup's whiskers with a brush on both sides (/3).

Sucking and swallowing were tested by placing a drop of feeding formula very close to the rabbit's snout/on the lips with a syringe; jaw movements and presence of regurgitation (ie, milk appearing in the nose) were observed (/3).

Head turning during feeding was evaluated together with the previous point and aimed to define the whole reaction to feeding (/3).

Surface righting reflex was scored as negative when the animal did not return to prone position after 2 seconds, when placed in supine position, out of 5 attempts (/5).

Odor aversion was tested placing a small sponge soaked in chlorhexidine 2−3 millimeters from the pup's snout (/3).

Pain sensation was tested by a pinprick on the hind limbs (/3).

The maximum score attainable in this section was 20 points.

Neuromotor patterns and maturation

This assessment was based on an adaptation of the protocol used by Mitchell et al to evaluate early developmental milestones in a rabbit model.[81] The pups were observed daily during the first 14 PNDs for a period of 90 seconds. The test was performed in an open field 30 × 30 cm, divided into a grid with 9 squares of 10 × 10 cm. The changes in 11 aspects of the rabbits' physical and neurobehavioral development were recorded.

- First day of eye opening.
- First sign of fur on the body.
- First day of head elevation for more than 1 minute.
- Body elevation: the first day of simultaneous standing on fore limbs and hind limbs
- Dragging: the immature locomotion pattern in rabbits; the pups move showing a distinct movement of the forelegs and dragging the hind limbs. The shift to the mature pattern was recorded when the locomotion mode transited to walking on 4 legs.
- Circling: another immature pattern of movement in newborn rabbits is characterized by crossing 1 fore limb over the other while the abdomen and the hind limbs, inactive, act as pivots, generating a movement in circle. The mature pattern was recorded on the first day of straightforward and non-circling locomotion.
- Hopping: a very characteristic pattern of adult rabbits' locomotion; it consists of moving the fore legs alternatively and the hind legs synchronously. Hopping is developed during time; therefore, the animals were scored as following: no hopping (0); 1−2 hops during the observation period (1); 3−9 hops during the observation (2); more than 10 hops (3); 50% of the movement was hopping (4); 4 corresponding to mature behavior. We thus recorded the first day that the animal got the maximum score.
- Falling: we considered as a fall any sudden lateral rotation of the body with 2 or more legs losing contact with the ground during locomotion. Mature behavior was recorded on the first day without any falls.
- Righting: the animal was placed in a supine position and righted itself back to prone position with at least both fore limbs touching the ground; this was performed 5 times. Mature behavior was defined if the pup righted itself within 2 seconds on all 5 attempts.
- Cliff avoidance: the pup was placed with its face and fore paws on the edge of a 5-cm high platform and observed for cliff avoidance. The pattern was considered as mature on the first day the animal used backward propulsion to avoid a cliff/sudden drop in the platform.
- Daily activity levels (DAL; total displacement) were scored as the number of squares crossed within the observation field during 90 seconds. We considered that the animal crossed a square when all 4 paws were in it.

Long-term evaluation was performed Open Field Behavioral Testing (OFBT) and Object Recognition Task (ORT) as described previously,[79] and by the T-maze (TM) and 8 radial-arm maze (RAM) tests for rabbits.

Open field test

Rabbits were evaluated for spontaneous behavior in an open field (OFT)[79] at 2 time points, PND 32 and PND 46. On the day prior to the test, animals were habituated to the testing room by placing the entire litter in an open field (150 × 150 cm, 80-cm height) for a period of 30 minutes. They were then returned to their cages.

The open field test was performed on a designated 80 × 80-cm area divided into 26 × 26 cm squares. Each rabbit was individually placed in a starting box (20 × 20 cm on PND 32; 25 × 25 cm on PND 46), which was removed after a maximum of 60 seconds. The animal was then left free to explore the open field over a period of 5 or 10 minutes on PND 32 and PND 46, respectively. Afterward it was returned to its cage. Again, each test was video recorded and evaluated a second time. The scoring grid is shown in Appendix 4.

We evaluated the rabbit's activity in the open field. We reported the delay before entering it, the time spent in the central area, and the time spent in corners (global activity). We also scored behavioral activity in the open field, through the number of escape attempts, and episodes of rearing, the time spent in self-grooming, and the presence or absence of digging, biting, urinating, and defecating behavior.

Novel object recognition test

Rabbits were evaluated for novel object recognition (NORT)[79] the day after the OFT, in the same setting.

Each animal was placed in the starting box for 30 seconds maximum before the test started. Each session was composed of 3 sections. During the sample phase, 2 boxes containing the same odor-based stimulus (apple) were placed in the field for 5 or 10 minutes (on PND 33 and PND 47, respectively). Afterward, in the retention phase, the rabbit was returned to the transport box for a 30-minute interval. Finally, during the testing or choice phase, 2 different odor-based stimuli (apple and orange) were presented to the animal for 5 or 10 minutes more, in a different position. At the end of the test, the rabbit was returned to its cage. The test was video recorded and scored later. The scoring grid is shown in Appendix 5.

In the sample phase, we evaluated the total time of interaction with the 2 stimuli (exploration). In the testing phase, we noted the same parameters for both the familiar (apple) (Tf) and the novel (orange) (Tn) stimuli. We thus calculated the discrimination index (DI) as follows:

$$DI = ((Tn - Tf))/((Tn + Tf))$$

For both phases, exploration of the object was considered when the rabbit showed sniffing, touching, and having moving vibrissae while directing the nose toward the object at a distance of less than 1 cm.

T-maze test

Rabbits were evaluated for working memory in a free-choice T-maze[82] at 2 time points, on PND34-36 and PND48-50. Each rabbit was tested once daily for 3 consecutive days.

The maze was composed of a common corridor and 2 goal arms (80 × 15 cm on PND34 and 80 × 20 cm on PND48). The 2 goal arms were not separated, so the animal could freely choose either of them. When the animal entered 1 of the 2 arms (ie, all 4 paws were into the arm), it was closed with a sliding gate to let the rabbit explore it and to prevent exploration of the opposite one. At the end of each arm, a visual cue was placed (a triangle and a square).

Each testing session was composed of a sample run and a testing run, with a 15-minute interval in between. The rabbit was placed individually in the starting box and confined for a maximum of 30 seconds before it was allowed to choose a goal arm. Once the animal had entered the common corridor, the starting box was closed. We considered that it had made a choice when all 4 paws were placed in the arm; at this point, the chosen arm was closed to prevent the access to the opposite one, and the pup was allowed to explore it for 30 seconds, then it was returned to the box for a 15-minute interval. If the animal failed in the sample run, that is, it did not choose any arm within 3 minutes from the starting box opening, a second sample run was allowed after 15 minutes. The testing run occurred with the same conditions described for the sample run, but no second try was allowed in case of failure. After each session, the animals were returned to their cage.

Again, each run was video recorded and scored a posteriori.

For this test, we evaluated the time spent in the starting area after its opening, total spontaneous alternation (expressed as %), and the failure rate in the 3 testing sessions.

Radial-arm maze test

Starting from PND 46 and for a period of 2 weeks, rabbits were evaluated on a radial-arm maze (RAM) test, patterned after multiple experiences with rats, mice, and other animals.[83–85]

The maze was composed of an octagonal central area giving access to radial arms 25 cm in width and 1 m in length.

The rabbits were tested every day in the early morning, before feeding. The test included a habituation period (1 daily session on 5 consecutive days, on PND 46–50), in which no food was available in the maze, followed by a retention phase of 2 days during which no run was performed. During the habituation, 3 rabbits at a time were allowed to enter the maze. At the beginning of each session, the animals were placed in the middle of the central area and were allowed explore the maze for 30 minutes. During that period, the examiners left the room so that the animals' behavior was not conditioned by human presence. During the following week (PND 52–56), the evaluation period was performed. It consisted of 5 more daily sessions, in which 1 cuboidal piece of apple was present in pots positioned at the end of each arm, invisible from the central area. During the evaluation period, the rabbits were tested individually, for 15 minutes. Each session was videotaped and evaluated a posteriori.

For each session, we evaluated the following: the number of consumed baits, the number of correct choices out of the first 8 choices (learning score); the total number of visits, the number of double entries (errors), and the number of arm entries until a choice was repeated.

Supplement 4: Euthanasia, Brain Harvesting, and Histology

After neurobehavioral testing (day 1 or day 56), rabbits were anesthetized with intramuscular ketamine (35 mg/kg) and xylazine (6 mg/kg) and transcardially perfused with 0.9% saline and heparin (100 μm/mL) followed by perfusion fixation with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The brain was removed to be immersion fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Whole-brain volumes, including cerebellum and brainstem, were determined using the fluid displacement method. After 72 hours, brains were paraffin embedded and subsequently serially sectioned.

Brains were serially sectioned at 4 μm from anterior to posterior, starting from the prefrontal cortex. Sets of 4 coronal sections were taken every 50 μm at 3 levels for further staining and analysis. Level 1 started in the prefrontal cortex, level 2 at the medial septal nucleus, and the level 3 at the beginning of the hippocampal formation. Staining was done with Cresyl Violet (Sigma). The primary antibodies used in these experiments included the following: mouse monoclonal anti-human Ki67 (Agilent); mouse anti-synaptophysin monoclonal

antibody SY38 (Abcam); polyclonal anti-Iba1 (Wako Chemicals). The secondary antibodies used were Alexa Fluor 488 conjugate (ThermoFisher). Degenerating nuclei were visualized by a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method for fluorescent in situ end labeling of double-stranded DNA fragmentation (Millipore). Sections were counterstained with Hoechst 33324 (Sigma-Aldrich).

Imaging acquisition and quantification

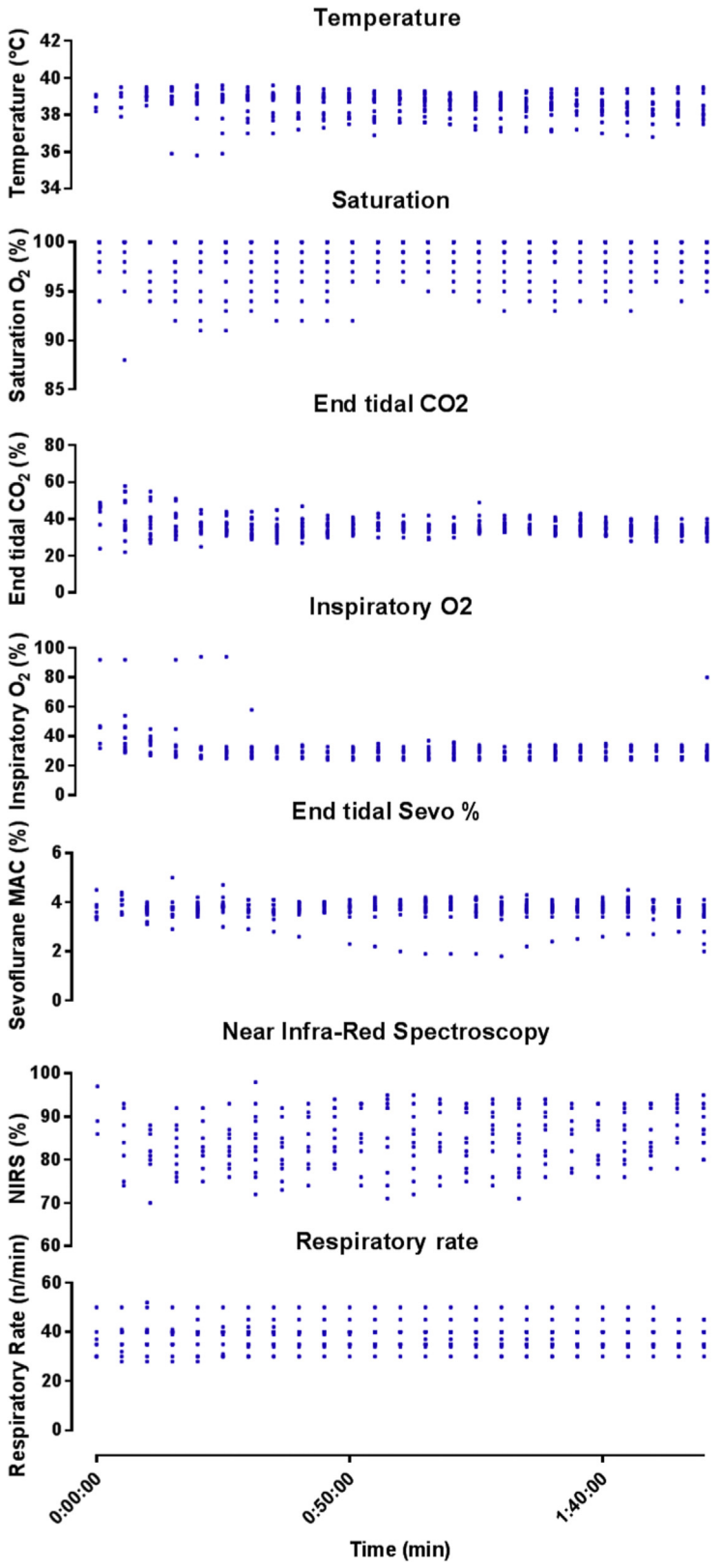
Histological slides were digitized using the Zeiss AxioScan Z1 imaging platform (AxioScan Slide Scanner, Carl Zeiss MicroImaging GmbH, Munich, Germany), using a 20× Plan Apochromat objective coupled to a 3 Chip CCD Camera (Hamamatsu Photonics, Japan). All focusing and field-of-view assembly was done by the Carl Zeiss Zen software (Carl Zeiss MicroImaging GmbH, Munich, Germany), which is integrated with the AxioScan device.

For neuronal densities, regions of interest (ROI) were selected on 4 consecutive slides for level 1 and 100 μm separated for levels 2 and 3. In each ROI, 5 different 100 × 100-μm squares were selected at low magnification so individual cells were not visible to avoid bias. In these squares, individual neurons were manually counted.

For quantification of the immunohistochemistry-positive cells in the TUNEL, Iba-1 and Ki-67 stains quantification profiles on the digitized whole-slide images were obtained with QuPath software[86] by using the fast cell counting and positive cell detection functions. Herein the whole region of interest was annotated, quantification was done, and positive cell counts were expressed as counts per area and/or percentage of positive cells per total cells detected.

For the mean fluorescence intensity quantification of the synaptophysin stain (Sy38), open source Fiji software (ImageJ) (http://fiji.sc/Fiji)[87] was used.

SUPPLEMENTAL FIGURE 1
Maternal anesthetic settings during surgery. Data are presented as individual data points, with mean and standard deviation



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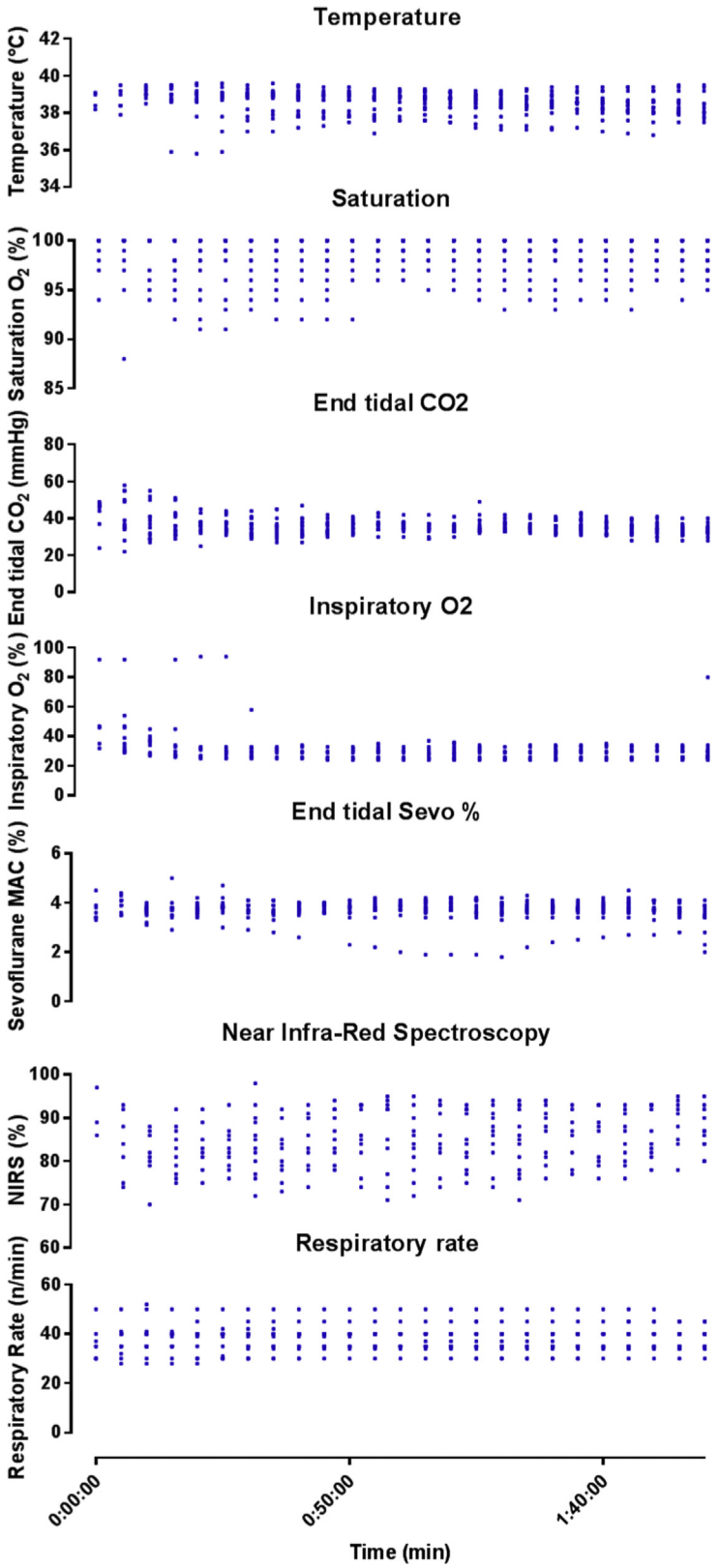
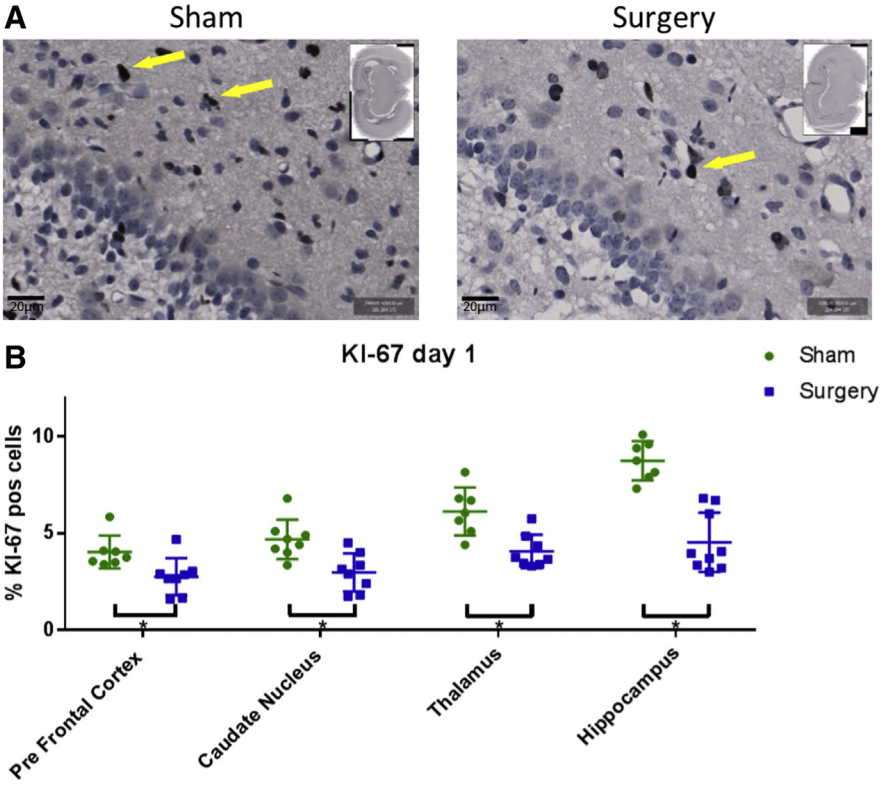
SUPPLEMENTAL FIGURE 2
Blood pressure and heart rate during the procedure. Data are presented as mean with standard deviation. *$P < .05$



Van der Veeken et al. Fetal brain development after maternal surgery. Am J Obstet Gynecol 2019.

SUPPLEMENTAL FIGURE 3

Proliferation on postnatal day (PND) 1. A, Representative image of the dentate gyrus in a sham (left) and surgery (right) animal stained with Ki67. The Ki67-positive cells are stained black as indicated by the yellow arrows. B, Percentage of Ki67-positive cells measured in the 4 regions of interest. Data are presented as individual data points, with mean and standard deviation. *$P < .05$



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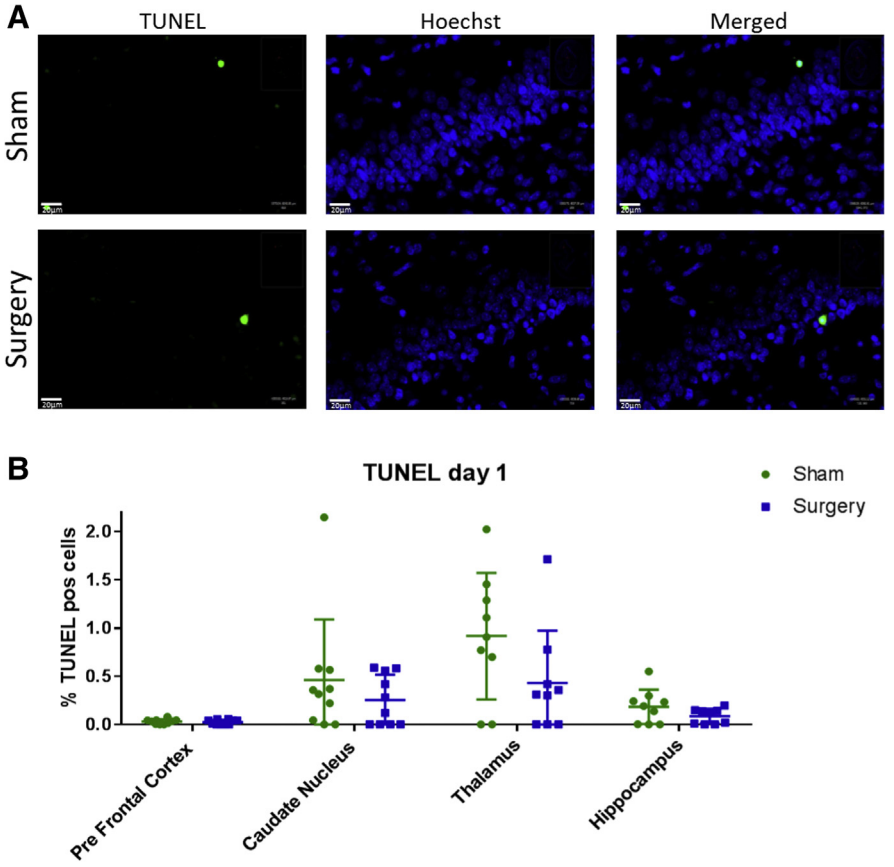
SUPPLEMENTAL FIGURE 4

Apoptosis on postnatal day (PND) 1. A, Representative image of the dentate gyrus in a sham (top) and surgery (below) animal stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). B, Percentage of TUNEL-positive cells measured in the 4 regions of interest. Data are presented as individual data points, with mean and standard deviation. *$P < .05$



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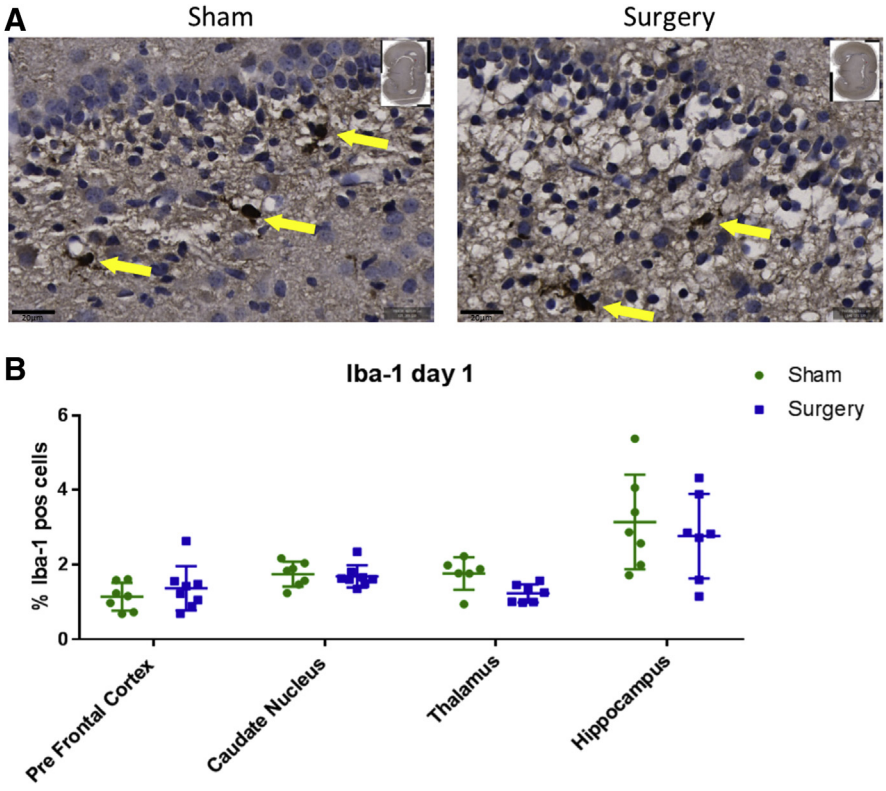
SUPPLEMENTAL FIGURE 5

Inflammation on postnatal day (PND) 1. A, Representative image of the dentate gyrus in a sham (left) and surgery (right) animal stained with Iba-1. The Iba-1 – positive cells are stained brown, as indicated by the yellow arrows. B, Percentage of Iba-1 – positive cells measured in the 4 regions of interest. Data are presented as individual data points, with mean and standard deviation. *$P < .05$



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