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

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Condensation: Maternal surgery during pregnancy has a negative effect on fetal brain development both histologic and neurobehavioral but with time these differences fade out.

Short title: Fetal brain development after maternal surgery

AJOG at a glance:

A. Why was the study conducted?

- Human data on the fetal impact of maternal surgery in pregnant women is lacking.
- Preclinical data on the impact of single and short exposure to anesthesia during pregnancy on fetal brain development is conflicting.
- Preclinical data on surgery during pregnancy is lacking.

B. What are the key findings?

- Surgery during pregnancy leads to lower neuron and synaptic density, and consequently to a motor and sensory deficit at birth.
- Animals develop slower
- These differences fade out over time.

C. What does this study add to what is already known?

- This study demonstrates short- and long- term effects of maternal surgery during pregnancy.
- This study shows how the impact evolves in time.

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

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Abstract

Background

Recently the US Food and Drug Administration called for cautious use of anesthetic drugs during pregnancy. In 0.2-2% of pregnancies non-obstetric surgery is being performed. The consequences of anesthesia during pregnancy on fetal development remain unclear and preclinical studies in relevant animal models may help elucidate that.

Objective

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

Study Design

This is a randomized, sham-controlled study in time-mated pregnant does at 28 days of gestation (term=31d), 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 of 1 MAC) sevoflurane for two 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 CO₂, pH, lactate) were continuously monitored. Sham controls consisted of seven does (74 pups) undergoing invasive hemodynamic monitoring for 2h without sedation. At term, does were delivered by cesarean section under ketamine-medetomidine sedation and local anesthesia. Pups either underwent motor and sensory neurologic testing followed by euthanasia at day one or daily neurodevelopment testing for two weeks and extensive neurologic assessment at 5 and 7

weeks (open field and object recognition test, T- 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 ; $p=0.004$) and sensory (16 ± 2 vs 15 ± 2 ; $p=0.005$) neurobehavioral scores and lower brain-to-body weight ratios ($3.7\% \pm 0.6$ vs $3.4\% \pm 0.6$; $p=0.001$). This was accompanied by lower neuron density in multiple brain regions (e.g. hippocampus 2617 ± 410 vs 2053 ± 492 neurons/mm²; $p=0.004$) with lower proliferation rates and less synaptophysin expression. Furthermore, surgery pups had delayed motor development during the first week of life, e.g. hopping appearing later (6 ± 5 vs 12 ± 3 d; $p=0.011$). Yet, by seven weeks of age, neurobehavioral impairment was limited to a reduced digging behavior and no differences in neuron density or synaptophysin expression was seen.

Conclusion

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

Introduction

Annually 0.2-2% of pregnant women in Europe and the USA undergo surgery during gestation¹⁻⁶. This might be for non-obstetrical indications, e.g. appendicitis, cholecystitis, or, more rarely, for fetal surgery, both often performed under general anesthesia (GA). In the last decade, there has been growing concern over the potential detrimental effect of anesthesia on the developing brain. Clinical studies have found conflicting results, finding either an altered neurodevelopmental outcome, abnormal behaviour and learning difficulties or showing no difference at all⁷. Mainly during the brain growth spurt, the developing brain appears to be extremely vulnerable to external factors, including anesthetic drugs⁸. 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 non-obstetric (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 gamma-aminobutyric acid (GABA) receptor agonists (e.g., 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, decreased synapse formation and lead to learning impairment and lower memory function^{11,12}. In pregnancy, most of these drugs will freely cross the placenta and lead to similar outcomes¹³⁻¹⁵. Until now, these studies exposed fetuses to maternal anesthesia only whereas clinically, general anesthesia is (almost) only administered to pregnant women for a surgical purpose. The additional effect of surgery has

not been well studied. This is required as surgery induces an inflammatory process, which in turn may interfere with brain development^{16,17}.

Herein 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 hypothesize that maternal anesthesia combined with surgery will lead to a decreased neurologic outcome with histologic changes at birth and that this will lead to behavioral and cognitive impairment on 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 12h 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 min, then placed on the operation table while maintaining sevoflurane anesthesia with a mask. Intravenous and arterial catheters were placed in the ear and an IV bolus propofol 6.5 mg/kg (Propovent, 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 left lateral decubitus. Normoventilation was obtained with an inspiratory oxygen concentration of 30%, using a closed-circuit respirator (FelixDual™; AirLiquide 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 (5cm), 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, enrofloxacin (10mg/kg, intravenous (IV)) (Baytril 5%, Bayer, Diegem, Belgium),

medroxyprogesterone acetate (9 mg/kg, SC) (Depo-Provera, Upjohn, Puurs, Belgium) and lidocaine 4 mg/kg (wound infiltration)(Xylocaine, AstraZeneca, Brussel, Belgium) were given. Animals were awakened after exact 120min of anesthesia and recovered in their cages. Sham animals were partially restricted in a cage (59x41x37). Under local anesthesia IV and IA lines were placed and the animals were monitored for 120 min (Supplement1). 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. They were daily monitored using the Rabbit Grimace Scale¹⁸ (Supplement1).

Delivery

Caesarean section on day 31 (term) was performed in both groups (sham and surgery) under ketamine/medetomidine sedation (ketamine, 15 mg/kg intra muscular (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 (Supplement1). Time between falling asleep and delivery was kept below 5 minutes. Pups were kept in an incubator and gavage fed. On day one pups were randomized to either a short (one day) or long term (56 days) group. The latter group were raised by cross fostering from day 1 (Supplement 2).

Neurobehavioral assessments

Short term motor assessment comprised of scoring gait, posture, locomotion, activity and tone. Afterwards the cranial nerves, pain response and righting reflex were tested for sensory evaluation as described before¹⁹. In the long-term group, a behavioral maturation score was assessed daily from day 1-14. Herein 11 aspects of their physical and

neurobehavioral development were recorded. Thereafter on day 32-36 and 46-56 an open field test (OFT), T-maze, novel object recognition task (NORT) and radial-arm maze assessment was done. All assessments were filmed and scored by an observer blinded to the treatment groups. 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 four μm every 50 μm as described before¹⁹. 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 labelling (TUNEL) method for fluorescent in situ end labelling of double-stranded DNA fragmentation (Apoptag S7110; Millipore, Billerica, MA, USA). Four relevant brain areas were evaluated, i.e. the prefrontal cortex, caudate nucleus, hippocampus and thalamus. Further details and quantification methods can be found in Supplement 4.

Statistics

Data was 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 unpaired students t-test or Mann Whitney test. A p-value <0.05 was considered significant. Blood pressure and heart rate were compared using a 2-way ANOVA. 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, additionally no fetal bradycardia or fetal death was noted (Supplement Figure1). Compared to the sham group (n=7), surgery rabbits had a higher pO₂ (85.9 ± 8.1 vs. 169.5 ± 57.2 mmHg; p=0.001: begin and 88.1±7.4 vs. 138.8±24.8 mmHg; p< 0.001 end) 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=0.04) (Table 1). Heart rates were similar and blood pressure only differed at 10 and 15minutes after the start of the procedure (Supplement 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 %; p=0.0001). Following randomization, 41 sham and 83 surgery pups were assigned to the short-term, 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 < 0.001) but comparable birth weight (51.4±7.8 vs 48.7±8.3g, p=0.07), resulting in lower brain-to-body weight ratio (3.7 (sham) vs. 3.2%; p=0.001) (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=0.007) (Figure 2 & video1). This was mainly attributable to a lower gait (p=0.01), locomotion (p=0.02) and motor activity of head (p=0.047), hind limbs (p=0.03) and front limbs (p=0.02). Additionally, lower sensory score

(16.6 ± 2.1 (sham) vs 15.3 ± 2.9 ; $p=0.01$) due to inability to suck and swallow ($p=0.02$) were seen in the surgery pups.

Neuropathological assessment noted 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 figure 4-5).

Long term outcome

On *neurodevelopmental assessment* during the first two weeks of life, surgery pups displayed a 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 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 %; $p=0.012$). Additionally, surgery rabbits displayed a lower exploration time in the NORT (54.9 ± 36.2 s vs. 23.1 ± 20.0 s; $p=0.005$). However no behavioral differences were noted between the two groups in the OFT. (Table 2)

At PND 46 surgery rabbits displayed less active digging behavior in the OFT (2/10 vs. 6/7; $p=0.015$). Yet, no other differences were noted in the OFT, NORT or T-maze testing. (Table 2)

At PND 53 and 56, four sham and five surgery rabbits underwent RAM testing. None of these rabbits consumed enough bait so no learning curve could be determined, neither any further analysis for this parameter.

On PND 56 body weights (1867 ± 265 vs. 1823 ± 190 g; $p=0,6$) and brain-to-body weight ratios ($0,51 \pm 0,07$ vs. $0,54 \pm 0,7$; $p=0,3$) were not different (Figure 1B). Overall mortality was comparable (4/16 vs. 2/19; $p=0.3$), no clear etiology for postnatal death could be determined for these cases. Upon *neuropathological assessment* no differences were noted in neuron densities and synaptophysin staining. (Figure 3B-4B).

Discussion

We studied the effects of a standardized laparotomy under GA on the developing fetal rabbit brain at a time point correlating to human mid-gestation. Compared to control equivalents, exposed pups displayed significant neonatal and early post-natal neurodevelopmental impairment. However, by pre-adolescence, 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 non-human primates (prenatal). Aiming to simulate a clinical scenario, we studied the effects of the combination of surgery and anesthesia. We anesthetized does at day 28 which is at the beginning of the brain growth spurt³², 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, e.g. IL-6, TNF α , IL-1 β), 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 animal which apparently induced a transient increase in heart rate and blood pressure, which, however, fell after into the normal range after 10 min (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. Also fetal hemodynamics remained most probably

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 yet a normal body weight. These gross anatomical findings coincided with reduced *proliferation*, as earlier demonstrated in rodents³⁵⁻³⁹. The observed reduction in proliferation taking place over the last three days of gestation (about 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 a day after the insult. Several underlying mechanisms were suggested, including direct neuronal excitation^{41,42} and exposure to pro-inflammatory cytokines⁴³⁻⁴⁶. In our study we did not study apoptosis or neurodensity immediately after the insult, because we primarily aimed for functional outcomes rather than mechanisms. Brains were investigated in the neonatal period, four days after the combined insult, when differences in apoptosis may no longer be measurable. Therefore, a profound elevation of apoptosis is not expected at this time period, yet these processes may have taken place. Apoptosis has been shown to be no longer demonstrable as early as 48h after exposure to dizocilpine²¹. Dizocilpine is a non-competitive NMDA antagonist⁴⁷, which is presumed to impact the fetal brain through a similar mechanism as sevoflurane⁴⁸. The presence of in utero deceased fetuses, which is not uncommon in rabbits, may also have an effect on neurodevelopment, e.g. by the release of inflammatory agents.^{16,17} In order to assess such effect, we repeated the statistics by

excluding the five does in which over half of the fetuses died, however found nearly the same differences between anesthesia exposed and control fetuses.

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 synapses activation and strengthening⁵¹⁻⁵³. Herein 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 inhibitory or excitatory, their location (axoaxonic, axodendritic or axosomatic) or the nature of the neurotransmitters involved.

Other mechanisms 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 gliosis⁵⁶.

This experiment also involved progressive sensory (day 1), motor (day 1 up to day 14) as well as neurocognitive (day 32-36 & 46-56) function testing. The most striking observation was that initial functional differences became smaller over time. In those animals followed-up longitudinally the effects were fading out with only one persistent difference, i.e. in digging behavior (46 days), coinciding with comparable histology in both groups. Digging is a pattern of mature behavior which rarely occurred at PND 32. The progressive decrease in impact contrasts with findings in rodents, in which, except for one study in pregnant mice⁵⁷, 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 indeed do '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, like humans and rabbits.⁶³ Notably, in terms of plasticity, the rodent and rabbit brain differ substantially⁶⁴. 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 humans⁶⁷.

In this experiment 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 man. The experiment was carried out at a timepoint when the brain is rapidly developing and the transition of neurogenesis and synaptogenesis are abundant. It remains unclear why in this model, rabbits seemed to "recover" from the neurobehavioral impairment. We speculate this may be due to neuroplasticity, a process that was earlier observed in rabbits with intra-uterine growth restriction⁶⁸. In the latter, the insult is chronic and fetuses are born with a lower birth weight, which may make them more vulnerable and 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. Actually, two months after the combined insult, some subtle (digging) differences were persisting.

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 findings⁶⁹. 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, have also 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 out of a 31-day gestation) and continuing after birth. Unlike in rats and mice (postnatal) or sheep and non-human primates (prenatal)³², which is relevant to the current research question³². The rabbit has frequently been used to study the effect of perinatal insults, including prematurity¹⁹, intraventricular hemorrhage^{70,71}, hypoxia⁶⁹, and infection¹⁶, on neurodevelopment. Despite being relatively immature at birth, rabbits show a large repertoire of spontaneous behaviors (motor activities and reflex responses) that are well characterized⁷²⁻⁷⁵ and can be reproducibly tested. Furthermore, rabbits have a placental structure (hemodichorial, i.e. containing two trophoblast layers) that is closer to that of

humans (hemomonochorial, i.e. one trophoblast layer) than that of rodents (hemotrichorial, i.e. three trophoblast layers)⁷⁶. This is relevant for the passage of drugs⁷⁷. Moreover, it is also considered suitable for long-term studies of perinatal insults^{65,68,78,79} through a foster caring system⁷⁸. The pups can thus be followed longitudinally far beyond the neonatal age.

Conclusion

Term pups, delivered from does exposed to two 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 two weeks of life, and poorer cognitive function and altered species-specific behavior at five weeks. However, at seven weeks of age neurologic function tests could not discriminate surgery from sham rabbits anymore, except that animals from operated mothers displayed an abnormal digging behavior.

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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 (mmHg)	28,0 ± 4,0	31,2 ± 4,7	30,4 ± 4,6	34,5 ± 4,9
pO ₂ (mmHg)	85,9 ± 8,1	169,5 ± 57,2	88,1 ± 7,4	138,8 ± 24,8
Lactaat (mmHg)	4,2 ± 2,7	3,7 ± 1,3	1,1 ± 0,4	4,4 ± 2,1

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 (y/n) (%)	0,0	29,4	0,06	71,4	30,0	0,2
Digging (y/n) (%)	25,0	11,8	0,6	85,7	20,0	0,015
Urinating (y/n) (%)	8,3	17,6	0,6	28,6	10,0	0,5
Defecating (y/n) (%)	16,7	29,4	0,7	28,6	50,0	0,6
Novel Object Recognition Test						
Exploration in sample run (sec)	54,9 ± 36,2	23,1 ± 20,0	0,005	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 (sec)	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	47,6 ± 37,8	36,7 ± 29	0,7

Extra material:

Figure 1: Biometrics: body weight, brain weight and brain-to-body weight ratio at A) PND 1, B) PND 56. Data are presented as individual data points, with mean and SD. Asterisk indicates $p < 0.05$.

Figure 2: Motor and sensory neurobehavioral outcome on PND 1. Data are presented as individual data points, with mean and SD. Asterisk indicates $p < 0.05$.

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 four regions of interest on PND 1. C) Neuron density in the four regions of interest on PND 56. Data are presented as individual data points, with mean and SD. Asterisk indicates $p < 0.05$.

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 four regions of interest on PND 1. C) Integrated density synaptophysin in the four regions of interest on PND 56. Data are presented as individual data points, with mean and SD. Asterisk indicates $p < 0.05$.

Figure 5: Neurodevelopmental patterns during the first two weeks of life. Hopping and dragging is presented as a Kaplan Meyer survival curve with the day on which a rabbit pup acquired a mature behavior, i.e. 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 SD. Asterisk indicates $p < 0.05$.

Supplemental Figure 1: Maternal anesthetic settings during surgery. Data are presented as individual data points, with mean and SD.

Supplemental Figure 2: Blood pressure and heart rate during the procedure. Data are presented as mean with SD. Asterisk indicates $p < 0.05$.

Supplemental Figure 3: Proliferation on PND 1. A) Representative image of the dentate gyrus in a sham (left) and surgery (right) animal stained with KI-67. KI-67 positive cells are stained black as indicated by the yellow arrows. B) Percentage of KI-67 positive cells measured in the four regions of interest. Data are presented as individual data points, with mean and SD. Asterisk indicates $p < 0.05$.

Supplemental Figure 4: Apoptosis on PND 1. A) Representative image of the dentate gyrus in a sham (top) and surgery (below) animal stained with TUNEL. B) Percentage of TUNEL positive cells measured in the four regions of interest. Data are presented as individual data points, with mean and SD. Asterisk indicates $p < 0.05$.

Supplemental Figure 5: Inflammation on PND 1. A) Representative image of the dentate gyrus in a sham (left) and surgery (right) animal stained with Iba-1. Iba-1 positive cells are stained brown as indicated by the yellow arrows. B) Percentage of Iba-1 positive cells measured in the four regions of interest. Data are presented as individual data points, with mean and SD. Asterisk indicates $p < 0.05$.

Supplemental Video 1: motor evaluation on PND 1. A: sham pup with synchronous movement of hind legs (=hopping). B: Surgery pup: hind legs are not used synchronously and falling is seen frequently.

ACCEPTED MANUSCRIPT

Supplement 1: Maternal manipulations: (457 words)

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 one 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, Connecticut, USA)⁸². Sevoflurane was continued during surgery at 1MAC (3.8%). Microultrasound (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 90min 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. Enrofloxacin 10mg/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 spontaneous respiration returned.

Animals assigned to the sham manipulation group were placed in a 59 x 41 x 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 min 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 enrofloxacin and medroxyprogesterone acetate as above.

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

During the remainder of the pregnancy, does were daily monitored using the Rabbit Grimace Scale¹⁸. Using this scale rabbits are observed for a couple of minutes and five action units are scored: orbital tightening, cheek flattening, nostril shape, whisker change & position and ear shape & position. Each item is scored: 0 (not present), 1 (moderately present) or 2 (obviously present).

Caesarean section 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 mL T61; Intervet International BV, Boxmeer, The Netherlands).

Supplement 2: manipulation of pups. (193 words)

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 x 25 cm). Pups were gavage fed twice daily with a Milk Replacer (FoxValley 30/50, Lakemoor Illinois, US) supplemented with probiotics and colostrum (FoxValley 30/50, Lakemoor Illinois, US) at 150mL/kg/feeding. At each feeding, their bladder was emptied by genital stimulation. On post-natal 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-nurse⁷⁹. A doe that did not undergo any kind of manipulation was housed in a double cage to which a nesting box (54 x 31 x 27 cm) was connected. The foster mother raised a mixed group of 8 to 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. 2000 words

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 before^{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 x 30 cm area divided in quadrants over a time period of 90 seconds. Each animal was left free to ambulate. Posture, gait, locomotion, motor activity of head, fore 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 travelled (/13).

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

Limb tone was tested by passive flexion and extension of one 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, that 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 kit'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 (i.e., 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 two seconds, when placed in supine position, out of five 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 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 model⁸³. 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 x 30 cm, divided in grid with nine squares of 10 x 10 cm. The changes of 11 aspects of their 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 one minute.
- Body elevation: the first day of simultaneous standing on fore and hind limbs
- Dragging: it is 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 four legs.

- Circling: another immature pattern of movement in new-born rabbits is characterized by crossing one 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: it is a very characteristic pattern of adult rabbits' locomotion; it consists in moving the forelegs 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 to 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 the animal got the maximum score.
- Falling: we considered as a fall any sudden lateral rotation of the body with two 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 kit righted itself within 2 seconds on all 5 attempts.
- Cliff avoidance: the kit 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 (total displacement; DAL) 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 four paws were in it.

Long term evaluation was performed Open Field Behavioral Testing (OFBT) and Object Recognition Task (ORT) as described previously⁷⁹, and by the T-maze (TM) and an 8 radial arm maze (RAM) tests for rabbits.

Open field test

Rabbits were evaluated for spontaneous behavior in an open field (OFT)⁷⁹ at two 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 x 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 x 80 cm area divided into 26 x 26 cm squares. Each rabbit was individually placed in a starting box (20 x 20 cm on PND 32; 25 x 25 cm on PND 46), that was removed after a maximum of 60 seconds. The animal was then left free to explore the open field over a time period of 5 or 10 minutes on PND 32 and PND 46 respectively. Afterwards it was returned to its cage. Again, each test was video recorded and evaluated in 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 of 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 three sections. During the sample phase, two 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). Afterwards, in the retention phase, the rabbit was returned to the transport box for a 30 minutes interval. Finally, during the testing or choice phase, two 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 two 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 towards the object at a distance of less than 1 cm.

T-maze test

Rabbits were evaluated for working memory in a free-choice T-maze⁸⁴ at two time points, on PND34-36 and PND48-50. Each rabbit was tested once daily for three consecutive days.

The maze was composed of a common corridor and two goal arms (80 x 15 cm on PND34 and 80 x 20 cm on PND48). The two goal arms were not separated, so the animal could freely choose any of them. When the animals entered one of the two arms (i.e. all four 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 minutes interval in between. The rabbits were placed individually in the starting box and confined for maximum 30 seconds before they were 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 four 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 minutes interval. If the animal failed in the sample run, i.e. it did not choose any arm within three 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, they 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 three testing sessions.

Radial-arm maze test

Starting from PND 46 and for a period of two weeks, rabbits were evaluated on a radial-arm maze test (RAM test), patterned after the multiple experiences with rats, mice and other animals⁸⁵⁻⁸⁷.

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

The rabbits were tested every day in the early morning, before feeding. The test included a habituation period (one daily session on five consecutive days, on PND 46 to 50), in which no food was available in the maze, followed by a retention phase of two days during which no run was performed. During the habituation, three 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 let 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 to 56), the evaluation period was performed. It consisted of five more daily sessions, in which one 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 number of consumed baits, the number of correct choices out of the first eight choices (learning score); the total number of visits, the number of double entries (errors), the number of arm entries until a choice was repeated.

Supplement 4: euthanasia, brain harvesting and histology (435 words)

After neurobehavioral testing (day1 or day 56) rabbits were anesthetized with intramuscular ketamine (35 mg/kg) and xylazin (6 mg/kg) and transcardial perfusion with 0.9% saline and heparin (100u/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 μ m from anterior to posterior, starting from the prefrontal cortex. Sets of 4 coronal sections were taken every 50 μ m at three levels for further staining and analysis. First level started in the prefrontal cortex, level two at the medial septal nucleus and the third level at the beginning of the hippocampal formation. Staining was done with Cresyl Violet (Sigma) and the primary antibodies used in these experiments included: 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 labelling (TUNEL)

method for fluorescent in situ end labelling 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 handled 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 four consecutive slides for level one and 100µm separated for level two and three. In each ROI, five different 100x100µm 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 done using QuPath software⁸⁸ by utilizing 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>)⁸⁹ was used.