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Prior infection exacerbates postoperative cognitive dysfunction in aged rats

Iris B. Hovens,^{1,2} Barbara L. van Leeuwen,² Csaba Nyakas,^{1,3} Erik Heineman,² Eddy A. van der Zee,¹ and Regien G. Schoemaker¹

¹Department of Neurobiology, GELIFES, University of Groningen, Groningen, The Netherlands; ²Department of Surgery and Surgical Oncology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; and ³Behavioral Physiology Research Unite, Department of Morphology and Physiology, Health Science Faculty, Semmelweis University, Budapest, Hungary

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Hovens IB, van Leeuwen BL, Nyakas C, Heineman E, van der Zee EA, Schoemaker RG. Prior infection exacerbates postoperative cognitive dysfunction in aged rats. *Am J Physiol Regul Integr Comp Physiol* 309: R148–R159, 2015. First published May 13, 2015; doi:10.1152/ajpregu.00002.2015.—Older patients may experience persisting postoperative cognitive dysfunction (POCD), which is considered to largely depend on surgery-induced (neuro)inflammation. We hypothesize that inflammatory events before surgery could predispose patients to POCD. When part of our aged rats developed *Mycoplasma pulmonis*, this presented the unique opportunity to investigate whether a pulmonary infection before surgery influences surgery-induced neuroinflammation and POCD. Male 18-mo-old Wistar rats that had recovered from an active mycoplasma infection (infection) and control rats (healthy) were subjected to abdominal surgery and jugular vein catheterization under general anesthesia (surgery) or remained naïve (control). In postoperative week 2, behavioral tests were performed to assess cognitive performance and exploratory behavior. The acute systemic inflammatory response was investigated by measuring plasma IL-6 and IL-12. In the hippocampus, prefrontal cortex and striatum, microglial activity, neurogenesis, and concentrations of IL-6, IL-12, IL1B, and brain-derived neurotrophic factor on postoperative day 14 were determined. Rats still showed signs of increased neuroinflammatory activity, as well as cognitive and behavioral changes, 3 wk after the symptoms of infection had subsided. Rats that had experienced infection before surgery exhibited a more generalized and exacerbated postoperative cognitive impairment compared with healthy surgery rats, as well as a prolonged increase in systemic cytokine levels and increased microglial activation in the hippocampus and prefrontal cortex. These findings support the hypothesis that an infection before surgery under general anesthesia exacerbates POCD. Future studies are necessary to determine whether the found effects are aging specific and to investigate the magnitude and time course of this effect in a controlled manner.

postoperative cognitive dysfunction; learning and memory; neuroinflammation; brain-derived neurotrophic factor; infection

AGE-ASSOCIATED SURGICAL COMPLICATIONS are an increasing concern in medical care, among them postoperative cognitive dysfunction (POCD). Large observational studies indicate that 10% to 15% of surgical patients aged over 60 experience POCD for more than 3 mo (36, 37). This persistent POCD is associated with a reduced quality of life, increased disability, and dependency on social services as well as an increased risk of mortality in the first year after surgery (37, 42, 55).

Although the pathogenesis of POCD is considered multifactorial (30, 46), there is evidence that inflammation is a key factor in POCD development (11, 22, 40, 60). In addition to a

local and systemic inflammatory response, surgery is associated with microglial activation and increased levels of pro-inflammatory cytokines in the brain (3, 11, 58, 63). The resulting neuroinflammation may impair neuronal function and cognitive performance (3, 65). Accordingly, the postoperative (neuro)inflammatory response is associated with POCD development, and several authors provided evidence for a causal relationship (3, 11, 19, 26, 40, 45, 60).

An exacerbated (neuro)inflammatory response to surgery has been hypothesized to account for the increased risk of POCD in older patients (3, 4, 12, 27). This hypothesis does not explain why only some older surgical patients are prone to develop POCD. It has been suggested that immune challenges and chronic low-grade inflammation during life cause age-related alterations in the immune environment of the body and brain (16, 32, 33, 35, 52). Life events, such as chronic inflammatory disease, infection, and physical trauma, may thus contribute to an exacerbated neuroinflammatory response to surgery and might therefore be associated with the susceptibility to POCD. Accordingly, preoperative depression, metabolic syndrome, and a secondary surgical intervention increase the risk of POCD (1, 28, 29, 36).

The aim of the current study is to investigate the influence preoperative infection on POCD. We studied the influence of pulmonary mycoplasma infection before surgery under general anesthesia in rats on postoperative behavior, markers for (neuro)inflammation and neuronal functioning [brain-derived neurotrophic factor (BDNF) and doublecortin-X (DCX)]. We hypothesized that this would exacerbate postoperative (neuro)inflammation and cognitive impairment in rats.

METHODS

Experimental animals and design. Seemingly healthy, aged (18 mo) male Wistar rats were obtained from a breeding colony of the Semmelweis University (Budapest, Hungary). The rats were housed in two separate rooms under controlled conditions: temperature $20 \pm 2^\circ\text{C}$ and humidity $50 \pm 10\%$, 12:12 light-dark cycle (lights off at 9:00 AM). They had ad libitum access to laboratory chow and water. All experiments were approved by the local animal experiment and welfare committee (Dier Experimenten Commissie, Groningen, The Netherlands).

A malfunction of the environmental control system led to increased temperatures ($\pm 24^\circ\text{C}$) and humidity ($>70\%$) in one of the animals rooms for approximately 1 wk. In this period the rats in this room developed symptoms of an active airway infection. All rats were then housed under strict isolation and a broad screening of the most common infectious agents for rats [Federation of Laboratory Animal Science Associations (FELASA)-screening] was performed, revealing that the rats were infected with mycoplasma. The diseased rats showed spontaneous recovery from disease symptoms within 3 wk.

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Considering our interest in the influence of prior disease history on POCD, this gave us a unique opportunity to investigate the hypothesis that an active infection before surgery under general anesthesia would lead to increased postoperative deterioration of cognitive performance.

The rats that did not experience the unfavorable environmental conditions were included in the experiment as healthy controls. FELASA screening of a sample ($n = 4$) of these rats revealed that these animals also had antibodies against mycoplasma, but none of them displayed any symptoms.

All diseased rats were checked twice weekly for symptoms of disease by recording changes in body weight and checking for the presence of disease symptoms (sneezing, coughing, and breathing sounds) as well as general signs of sickness. As a measure for the severity of symptoms we devised a symptom score where 0 = no symptoms, 1 = occasional sneezing, 2 = regular sneezing, occasional coughing and/or breathing sounds, and 3 = regular sneezing, severe coughing and/or breathing sounds. The scores were added up during the total period that the animals were being monitored and average symptom scores over time were determined. All rats recovered spontaneously from the infection without medical or pharmacological treatment.

Rats that developed an active mycoplasma infection and had recovered spontaneously and healthy control rats were subjected to surgery under general anesthesia or remained naïve as control, leading to four experimental groups: healthy control (HC, $n = 12$); healthy surgery (HS, $n = 12$); infection control (IC, $n = 12$); infections surgery (IS, $n = 12$). After a 9-day recovery period the rats underwent several behavioral tests and were terminated on postoperative day 14.

Surgery. Surgery, mimicking major abdominal surgery in humans, was performed as described before (26). While the rats were under general anesthesia (3% sevoflurane in O_2 at 0.7 l/min) and analgesia (buprenorphine, 0.003 mg/kg subcutaneous), the intestines were exteriorized. The upper mesenteric artery was cleaned from surrounding tissue and clamped for 30 min, leading to temporary hypoperfusion of the mesenteric vascular bed (41). During this abdominal surgery, animals were equipped with a jugular vein catheter as described before (54). Control animals did not receive any surgical intervention, anesthesia, or analgesia.

Behavioral tests. The behavioral test protocol was performed as described before (26). An open field test was performed to assess exploratory and anxiety-related behavior on postoperative day 9. Behavior in a square novel arena (100 × 100 × 40 cm) was recorded for 5 min and analyzed using Ethovision (Noldus Information technology, Wageningen, The Netherlands) for total distance moved and the percentage of time spent in the corners of the open field (four times 20 × 20 cm), as well as for time spent on exploratory behavior (sniffing, rearing, and walking), anxiety-related behavior (scanning, pressure breathing, and freezing), grooming, and resting.

Visual and spatial short-term memory were studied in the novel object and novel location test on postoperative day 10. Rats were habituated two times 5 min to a test box (50 × 50 × 50 cm). After another 3 min habituation period, the test consisted of three phases of 3 min separated by a 45-s pause in which the rat remained in the test box. In the exploration phase, the rat was presented two identical objects (stacked Lego cubes or plastic bottles). In the novel object recognition phase (NO) the rat was presented with one familiar object and one novel object. In the novel location recognition phase (NL), the familiar object of the NO was returned to its original location, whereas the novel object of the NO was placed at a novel location. Behavior was recorded, and the time spent on object exploration was determined using Eline software for each test phase. The ratio of time the animal spent exploring the novel or relocated object compared with the total object-exploration time was taken as a measure of object or location recognition.

Spatial learning, memory, and cognitive flexibility were assessed in the Morris water maze (MWM). The maze (140 cm Ø, water of 26 ±

1°C) was divided in four quadrants. A platform was located in the target quadrant just below the water surface. Spatial cues were provided around the pool. Rats underwent training in the MWM on postoperative day 11 and 12. The training consisted of five training sessions, three training sessions on day 11 with a 1-h interval, and another two training sessions on day 12. Each training session consisted of three trials, in which the rat was sequentially placed in the quadrants without the platform and allowed to search for the platform. The trials stopped when the rat had located the platform and sat on it for 10 s. If the rat did not find the platform within 60 s, it was guided manually to the platform location and left there for 10 s. After completion of the training session, rats were towel dried and returned to their home cage. Average escape latencies during the training sessions were determined as measure for spatial learning.

A probe trial was performed on postoperative day 12, 24 h after the first training session, to assess spatial memory after a relatively short training period (three training sessions). A second probe trial was performed on postoperative day 13 to assess spatial memory after an extended training period (five training sessions). During the probe trials, the platform was removed from the maze. Rats were allowed to explore the maze for 60 s. The distance moved and the time spent in each zone during the probe trial were determined using Ethovision (Noldus Information Technology).

One hour after the second probe trial, on day 13, rats underwent reversal training consisting of four consecutive trials that were in all aspects similar to the training trials, except that the platform was located in the opposing quadrant. Average escape latency during the reversal training was determined as a measure for cognitive flexibility.

ELISA for inflammatory factors in plasma and brain tissue. Blood was sampled from the tail vein at baseline and from the jugular vein catheter 2, 6, 24, and 48 h after surgery from HS and IS rats. Additionally, a blood sample was taken by cardiac puncture in all animals, immediately before the rats were terminated. Blood was centrifuged for 10 min at 2,600 g, and plasma was collected and stored at -80°C. According to manufacturer's instructions plasma IL-6 (IL-6 Rat Elisa-kit Invitrogen, Frederick, MD) and IL-12 (IL-12 Rat Elisa-kit Invitrogen) were determined.

Animals were terminated on postoperative day 14 under deep anesthesia (2 ml/kg 6% pentobarbital sodium) by transcardial perfusion with saline containing 0.1% EDTA. Brains were collected and the hippocampus (HIP), prefrontal cortex (PFC), and striatum (STR) were dissected from one hemisphere of each brain. Brain tissue was homogenized in a 50 mM Tris-HCl buffer (150 mM NaCl, 0.002% Tween-20) containing Complete Mini protease inhibitor (Roche Diagnostics, Indianapolis, MN) and sonicated for 5 s twice. Homogenates were centrifuged for 15 min at 12,000 rpm and supernatant was collected. The supernatant was diluted to 5 mg/ml protein based on a Bradford assay. According manufacturer's instructions IL-6, IL-1B (IL-1B Rat Elisa-kit Invitrogen), and IL-12 p70 (IL-12 p70 Rat Elisa-kit Invitrogen) concentrations were determined.

Western blot for brain-derived neurotrophic factor in brain tissue. Supernatant of the HIP, PFC, and STR were collected as described above and diluted to 2 mg/ml protein in 50 mM Tris-HCl buffer (150 mM NaCl, 0.002% Tween-20) containing protease and phosphatase inhibitor and 25% lithium dodecyl sulfate (LDS) sample buffer (NuPAGE, Life Technologies, Carlsbad, CA). Samples were denatured for 10 min at 70°C, 10% reducing agent was added, and 20 µl of each sample was loaded into precast gels (NuPAGE 4–12% Bis-Tris gel, Life technologies). Proteins were separated by gel electrophoresis and transferred onto membranes (iBlot, Life technologies). Membranes were blocked with 5% BSA for 1 h at room temperature, incubated with 1:1,000 rabbit-anti-brain-derived neurotrophic factor (BDNF) (Alomone labs, Jerusalem, Israel) or 1:1,000,000 mouse-anti-β-actin (MP Biomedicals, Irvine, CA) in 5% BSA overnight at 4°C, and incubated with anti-rabbit or anti-mouse secondary antibody in 2.5% BSA for 2 h at room temperature. Between steps the membranes were thoroughly rinsed with Tris-buffered saline containing 0.1%

Tween. Protein bands were visualized by enhanced chemiluminescence (Thermo Scientific, Rockford, IL) and quantified using Image Lab (version 2.0.1, Bio-Rad Laboratories) to determine BDNF-to-actin ratios.

Immunohistochemistry for microglial activity and neurogenesis. After transcardial perfusion, one hemisphere of each brain was immersion fixed in 4% paraformaldehyde for 4 days, dehydrated with 30% sucrose in 0.01 M PBS, deep frozen, and cut into 30- μ m sections. Sections were stored in 0.01 M PBS with 0.1% sodium-azide at 4°C. Sections were pretreated for 20 min with 3% H₂O₂. To visualize microglia, sections were incubated for 3 days with 1:2,500 rabbit-anti-ionized-binding adaptor protein (IBA)-1 (Wako, Neuss, Germany) in 2% BSA, 0.1% Triton-X at 4°C, and incubated for 1 h with 1:500 goat-anti rabbit secondary antibody (Jackson, West Grove, PA) at room temperature. To visualize newly formed maturing hippocampal neurons, sections were blocked for 1 h with 5% normal rabbit serum (NRS), incubated with 1:1,000 goat-anti-doublecortin X (DCX) (Santa Cruz, Dallas, TX) in 1% BSA, 1% NRS for 3 h at 37°C, overnight at room temperature, and three nights at 4°C, and incubated overnight with 1:500 rabbit-anti goat secondary antibody (Jackson) at 4°C. The stainings were finished by incubating sections for 2 h at room temperature with avidin-biotin peroxidase complex (Vectastain ABC kit, Vector, Burlingame, PA) and 3,3'-diaminobenzidine (DAB) labeled using a 0.075 mg/ml DAB solution activated with 0.1% H₂O₂. All dilutions were made in 0.01 M PBS. All sections were thoroughly rinsed with 0.01 M PBS between staining steps. Sections were mounted on glass and dehydrated through gradients of ethanol and xylol solutions.

Stainings were analyzed in a blinded fashion in three sections per area for each animal. With the use of image analysis software (Image-Pro Plus 6.0), the number of microglia, the average total cell coverage, average cell body coverage, and average coverage by dendritic processes were determined in the dentate gyrus inner blade (DGib), Cornu Ammonis (CA)1 region, and CA3 region of the hippocampus, medial prefrontal cortex (PFC, Zilles's Cg1), and dorsomedial striatum (DMSTR). Since microglia activation is characterized by an increased cell body size and shortening of the dendritic processes, the cell body-to-total cell size ratio was used as a measure of microglia activation (25).

The number of DCX-positive cells in the dentate gyrus (DG) was determined by counting the number of DCX-positive cells in the granular layer of the DG using a light microscope at $\times 50$ magnification and corrected for the length of this layer (6). The outcome was

considered a measure for the number of newly formed neurons in the DG and thus neurogenesis.

Statistical analysis. Previously acquired data on MWM performance of young adult rats, and an expected aging effect, led to the assumption that healthy aged rats would have a dwell time of 32% in the target quadrant of the MWM, while surgery would reduce the dwell time to 25%, with a SD of 6%. A power analysis performed using G*power 3.1.9 indicated that to reach a power of 0.8 using a univariate ANOVA with four groups, a total sample size of 48 ($n = 12/\text{group}$) was required.

Data are displayed as means \pm SD. Statistical analysis was performed using SPSS 22.0 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY; IBM). Effects were regarded statistically significant when $P \leq 0.05$. Main effects of surgery and infection history, and infection history \times surgery interaction effects were determined using univariate two-way ANOVA or repeated measures ANOVA, when appropriate. In case of significant interaction effects, group differences were analyzed using ANOVA followed by Tukey HSD post hoc analysis. The time spent in the target quadrant and opposing quadrant in the MWM probe trials was analyzed using a three-way ANOVA with surgery, infection history, and quadrant as independent variables. In case of significant interaction effects, the time in the target quadrant was compared with the time in the opposing quadrant using a one-way ANOVA with Tukey HSD post hoc analysis.

Cognitive test outcomes and levels of inflammatory markers and BDNF were correlated using Pearson's correlations with a Bonferroni corrected significance level of $P = 0.002$.

RESULTS

Body weight and severity of symptoms. From the day the symptoms were first recognized, there was an overall increase in the symptom severity that subsided after approximately 10 days (Fig. 1A). Symptoms were not significantly present anymore from *day 6* before surgery, with only two rats that were still sneezing occasionally. Although symptom severity varied among rats, all rats displayed symptoms of mycoplasma infection. The healthy control rats did not show any of these symptoms.

Rats weighed 552 ± 13 g at arrival. Body weight of rats that developed an active infection (~ 28 days before surgery) declined significantly ($F_{7,144} = 4.68$, $P = 0.000$) but increased

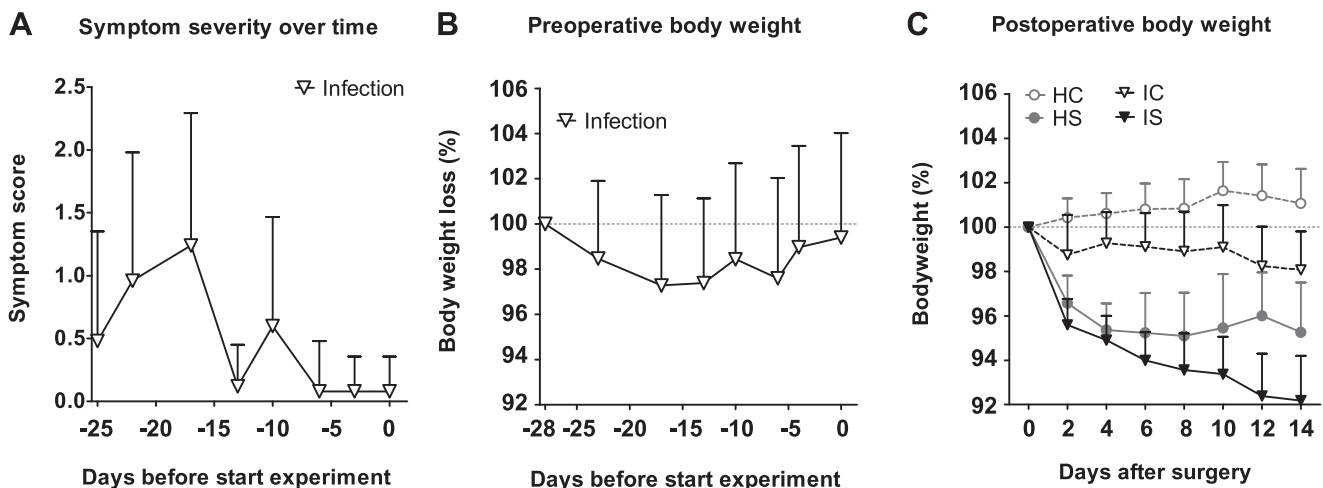


Fig. 1. Symptoms and body weight during mycoplasma infection. A: average symptom score over time as measure for symptom severity in rats that suffered from infection. B: preoperative body weight (as percentage from first body weight measurement) in rats with and without symptoms of infection. C: postoperative body weight as percentage from body weight on the surgery day. HC, healthy control; HS, healthy surgery; IC, infection control, IS, infection surgery

again from *day 13* before surgery (Fig. 1B). Healthy control rats were weighed starting at 2 wk before surgery and showed a significant body weight gain in this period ($2 \pm 2\%$).

After surgery, body weight declined significantly ($F_{7,308} = 60.48, P = 0.000$), and the postoperative change in body weight was significantly affected by infection history ($F_{7,308} = 20.87, P = 0.000$). One rat developed complications after surgery and was excluded from the experiment.

Behavior. Behavior in the open field is depicted in Fig. 2. Exploratory behavior in the open field was significantly affected by infection history and intervention. Anxiety-related behavior in the open field was significantly affected by infection history, as was the time spent in the corners of the open field (infection $67 \pm 10\%$ vs. healthy $56 \pm 12\%$, $F_{1,44} = 13.53, P = 0.001$). Resting behavior was significantly affected by infection history and intervention. In addition, there was a history-intervention interaction, where the animals with a history of active infection showed increased resting behavior following surgery. During the exploration phase of the novel object and location test, there was no preference for either the left or the right object in any of the groups, with an average exploration of $49 \pm 12\%$ of the left object compared with the total object exploration time. During all phases of the test, there were no significant group differences in either the time spent on exploration of the cage or the time spent on exploration of the objects.

One animal was excluded from the analysis of the novel object and location test because it hardly explored the objects ($<2\%$ total object exploration). Object recognition (Fig. 3A) was significantly affected by surgery and history \times surgery, where surgery led to impaired object recognition in rats with prior infection. Spatial recognition (Fig. 3B) was impaired by surgery in all rats but was not affected by infection history or infection history \times surgery interaction.

Two rats were removed from MWM analysis since they showed agitated/anxious behavior. The escape latency during MWM training (Fig. 3C) was significantly affected by training, indicating that overall rats improved in finding the platform. There was a significant effect of infection history and surgery, with the worst performance in rats that received surgery after infection.

Spatial memory in the MWM (Fig. 3D) in the first probe trial, after a relatively short training period, was not significantly affected by any of the experimental conditions. However, there was a significant effect of quadrant ($F_{1,87} = 16.27, P = 0.000$) and quadrant \times history \times intervention ($F_{1,87} = 4.11, P = 0.046$). Only HC spent a higher amount of time in the target quadrant compared with the opposing quadrant. During the second probe trial, after a longer training period, there was a significant effect of infection history \times quadrant ($F_{1,87} = 58.69, P = 0.000$), with only the healthy rats spending more time in the target compared with the opposing quadrant.

The average escape latency during the MWM reversal training, as measure of cognitive flexibility (Fig. 3E), was significantly affected by infection history. There were no history or surgery effects on the distance moved during the MWM tests (data not shown).

Systemic inflammation after surgery. Baseline plasma IL-6 concentrations did not differ between the surgery groups (HS: 1.8 ± 3.1 pg/ml, IS: 0.7 ± 0.5 pg/ml). There was no overall effect of infection history on systemic IL-6 and IL-12 concentrations after surgery (Fig. 4, A and C). Although IC and IS did not have significantly different cytokine concentrations 48 h after surgery (IL6: HC = 20 ± 8 , HS = 25 ± 14 ; IL-12: HC = 258 ± 74 , and HS = 84 ± 101), we observed an effect of infection history on the levels of these cytokines 14 days after surgery. At this time, there was a significant effect of infection history, surgery, and infection history \times surgery on plasma IL-6, with an increased IL-6 concentration in IS only (Fig. 4B). There was a significant effect of surgery on plasma IL-12 concentrations and a trend for an effect of infection history. Although IL-12 appears to be increased mainly in IS, there was no significant history \times surgery interaction effect (Fig. 4D).

Neuroinflammation after surgery. In the hippocampus, IL-1B concentrations (Fig. 5A) were significantly affected by infection history and infection history \times surgery, with only a significant difference between HC and both infection groups. In the frontal cortex, none of the conditions significantly affected IL-1B concentrations. In the striatum only infection history significantly affected IL-1B concentrations.

In the hippocampus neither infection history nor surgery influenced IL-6 concentrations significantly (Fig. 5B). IL-6

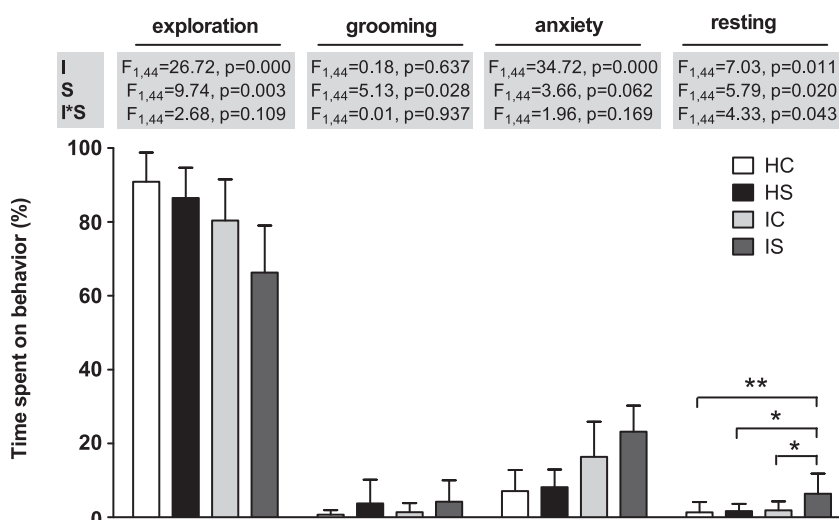


Fig. 2. Behavior in the open field. The percentage of time spent on exploration, grooming, anxiety-related behavior, and resting behavior in the open field are shown. I, infection history; S, surgery; T, time; HC, healthy control; HS, healthy surgery; IC, infection control; IS, infection surgery. * $P < 0.05$, ** $P < 0.01$.

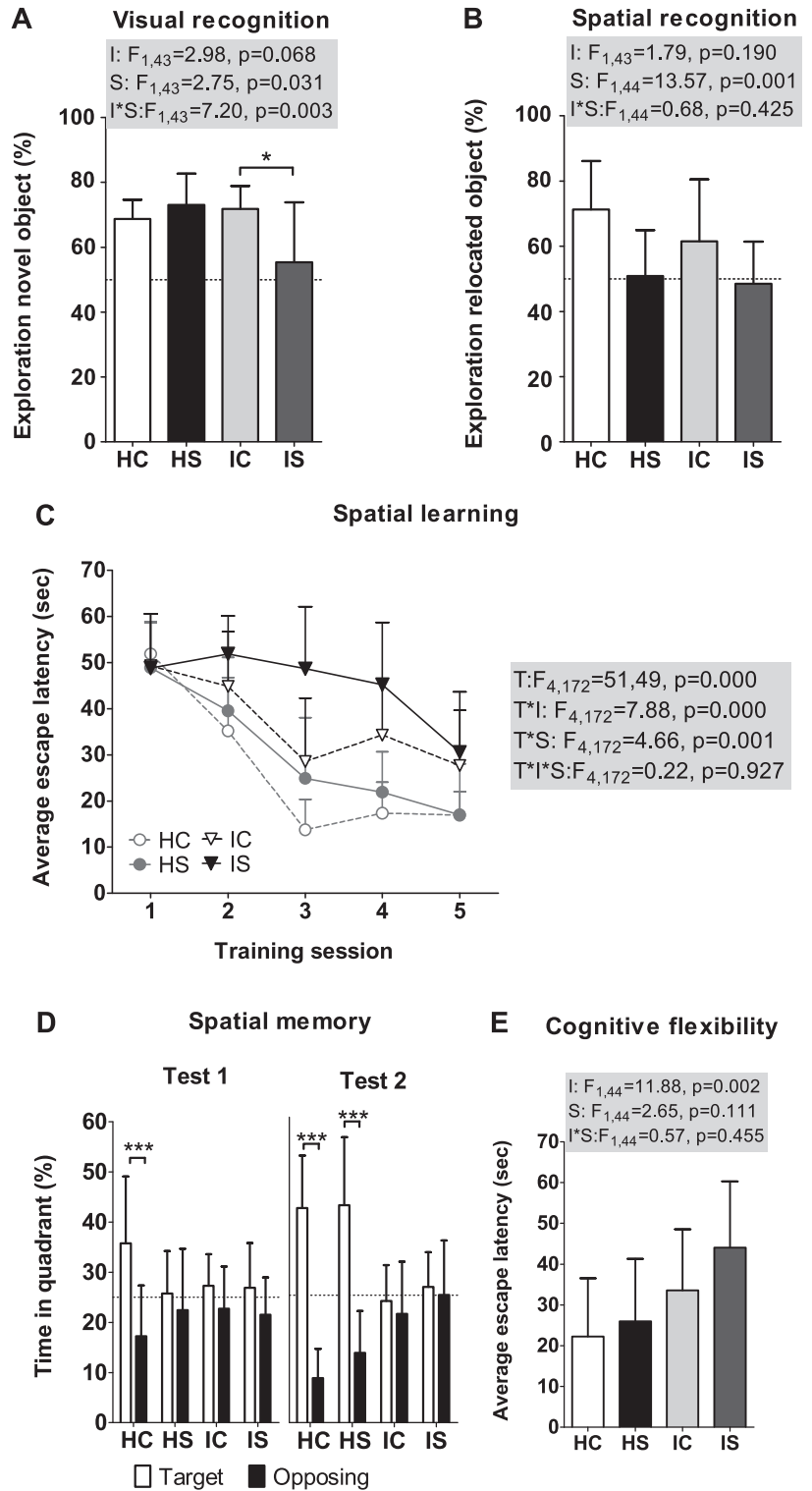


Fig. 3. Effects of prior infection and surgery on cognition. *A*: visual recognition in the novel object test. *B*: spatial recognition in the novel location test. *C*: spatial learning in the MWM. Average escape latency (in s) is shown for the 5 training sessions in the maze. *D*: spatial memory in the MWM after 3 (*test 1*) and 5 (*test 2*) training sessions. The time in the target quadrant (%) is shown. *E*: cognitive flexibility in the Morris water maze (MWM). Average escape latency (in s) during the four MWM reversal trials. Dotted horizontal line, reference line for expected exploration time (50%) or time in target quadrant (25%) if rats explore randomly. * $P < 0.05$, *** $P < 0.001$.

concentrations were significantly affected by infection history in the frontal cortex and striatum. IL-12 concentrations were affected by infection history in all three analyzed brain areas (Fig. 5C).

Microglial cells (Fig. 6) of HC rats seemed to be largely quiescent with round cell bodies and extensive dendritic processes. In the hippocampus, the cell body-to-cell size ratio, as

measure for microglial activation, was significantly affected by infection history, surgery, and infection history×surgery. Microglial activation was increased in IS compared with the other groups. In the prefrontal cortex, microglial activation was significantly influenced by infection history and surgery. Microglial activation in the dorsomedial striatum was not significantly affected by any of the conditions. Both prior infection

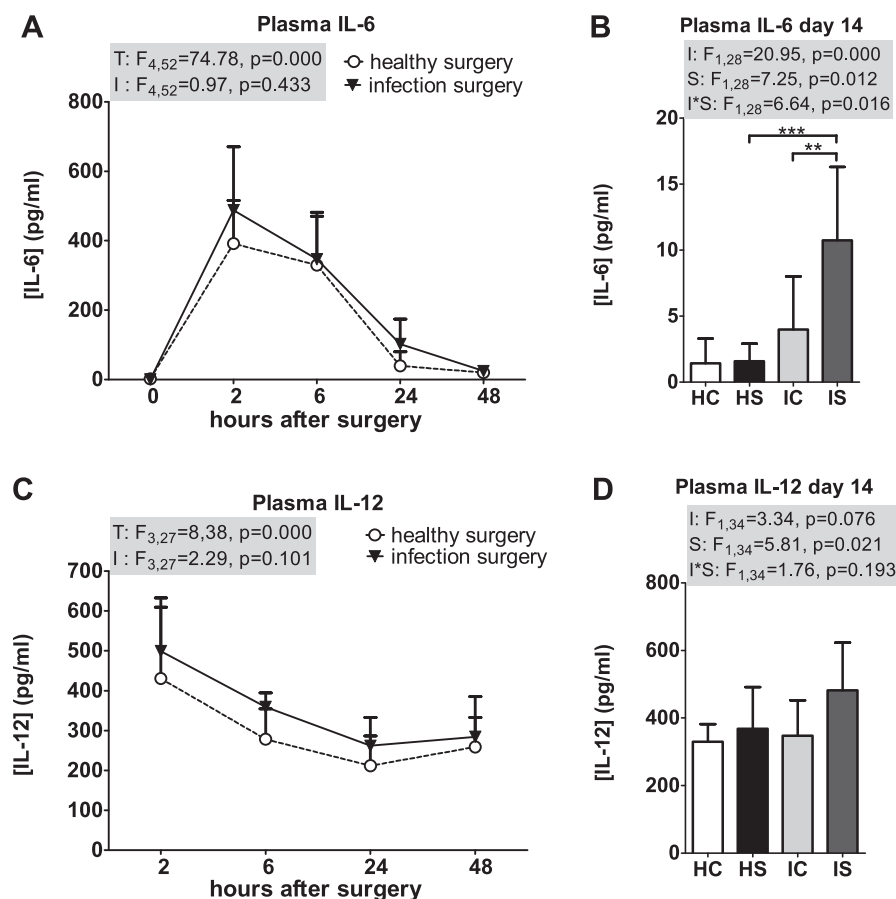


Fig. 4. Systemic inflammatory response. A: plasma IL-6 concentrations (pg/ml) at baseline (0) and in the first 48 h after surgery, determined in 8 rats/group. B: plasma IL-6 concentrations (pg/ml) on postoperative day 14. C: plasma IL-12 concentrations (pg/ml) in the first 48 h after surgery. Because of limited availability of plasma at baseline, plasma IL-12 concentrations could not be determined at this time point. D: plasma IL-12 concentrations (pg/ml) on postoperative day 14. ** $P < 0.01$, *** $P < 0.001$.

and surgery were associated with a more elongated shape of the microglial cell bodies, with the most marked changes in the IS group. This was not generally reflected in the average cell body size, which was only significantly increased in the hippocampus of the infection groups ($F_{1,40} = 9.50, P = 0.004$), but not influenced by other parameters or in other brain areas. Visual estimation of the dendritic processes indicated that arborization was mainly influenced by surgery in the hippocampus and prefrontal cortex, which was substantiated by measurements of the size of the dendritic processes (hippocampus: $F_{1,41} = 15.08, P = 0.000$; prefrontal cortex: $F_{1,41} = 28.75, P = 0.000$; dorsomedial striatum: not significant). The number of microglia was affected by surgery in the hippocampus (HC: 23 ± 2 ; HS: 24 ± 2 ; IC: 22 ± 2 ; IS: 24 ± 2 ; $F_{1,41} = 10.50, P = 0.002$) and prefrontal cortex (HC: 25 ± 3 ; HS: 28 ± 4 ; IC: 23 ± 2 ; IS: 30 ± 5 ; $F_{1,41} = P = 0.000$) but not the striatum (HC: 25 ± 5 ; HS: 26 ± 4 ; IC: 28 ± 10 ; IS: 25 ± 4 ; not significant).

Microglial activity in the hippocampus was significantly correlated with resting behavior ($r = 0.482, P = 0.001$). There were no other significant correlations between inflammatory markers and behavioral test outcomes.

Neuronal functioning after surgery. In the hippocampus, there was an effect of surgery on BDNF-to-actin ratio but not of infection history or infection history \times surgery (Fig. 7A). In the frontal cortex there was a significant effect of infection history on BDNF-to-actin ratio. Although there were no significant interaction effects in these regions, IS appears to have the lowest BDNF levels. In the striatum there was a main effect of infection history on BDNF-to-actin ratio. Interestingly,

BDNF-to-actin ratios were increased in rats that had recovered from an active infection.

All rats had a very low number of DCX-positive cells in the DG (Fig. 7C). The number of DCX-positive cells was significantly reduced by infection history and surgery.

DISCUSSION

When part of our aged rats developed symptoms of mycoplasma infection, this serendipitous event provided us with the opportunity to investigate whether a pulmonary infection in the period before surgery may influence surgery-induced neuroinflammation and POCD development. The findings of this study indicate that after the symptoms of infection had subsided, aged rats still showed signs of neuroinflammation, as well as cognitive and behavioral changes. When these rats were subjected to surgery under general anesthesia, they exhibited a more severe and generalized postoperative cognitive impairment. Additionally, 2 wk after the surgical intervention, increased systemic cytokine levels were still observed, as well as increased microglial activation in the hippocampus and prefrontal cortex, which may indicate persistent (neuro)inflammation.

Prolonged inflammatory and behavioral changes after infection. From the onset of the symptoms of a pulmonary infection, rats displayed a rapid increase in symptom severity and weight loss. Symptoms peaked after ~ 1 wk, before they resolved and body weight recovered to baseline levels, indicating recovery from infection. However, several weeks later,

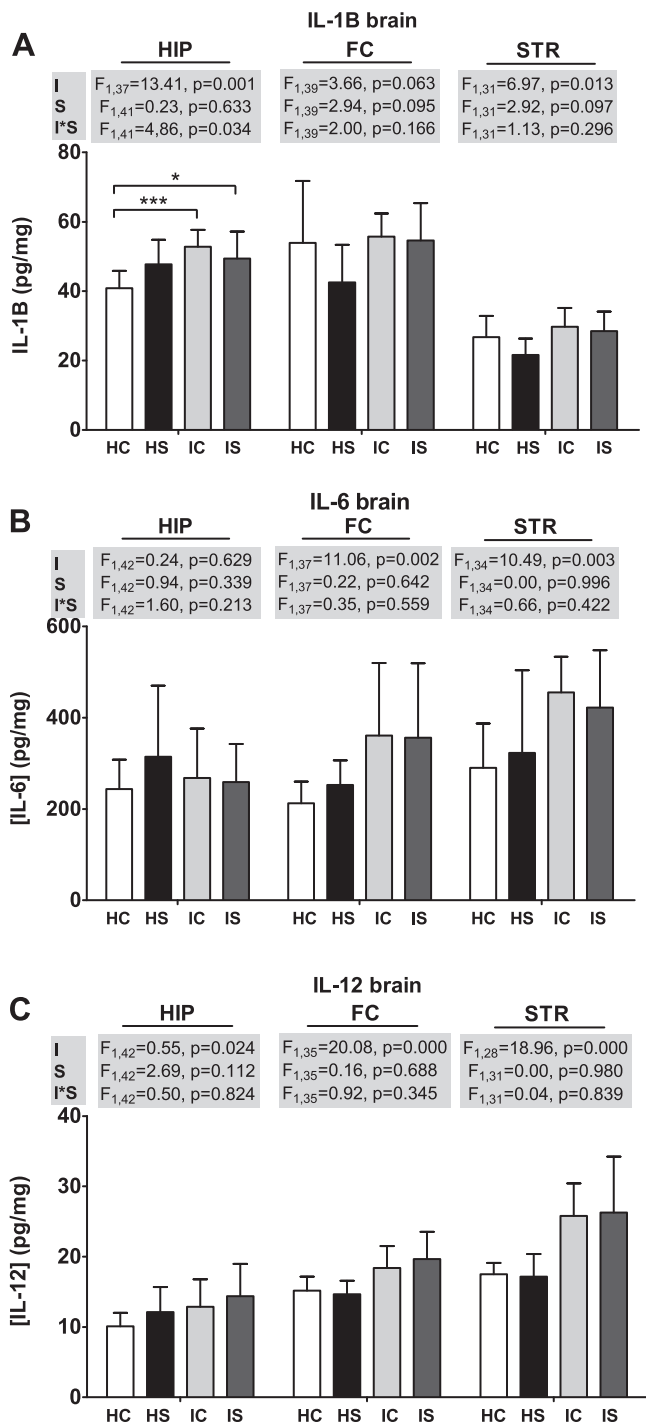


Fig. 5. Inflammatory factors in the hippocampus (HIP), frontal cortex (FC), and striatum (STR) of the brain. A: IL-1B concentrations (pg/mg protein); B: IL-6 concentrations (pg/mg protein); C: IL-12 concentrations (pg/mg protein). * $P < 0.05$, *** $P < 0.001$.

IC rats still showed signs of increased (neuro)inflammatory activity, with slightly increased plasma IL-6 concentrations, increased IL-6 and IL-12 levels in the frontal cortex and striatum, elongated microglial cell bodies, and signs of increased microglial activation in the hippocampus. Additionally, we observed alterations in exploration and anxiety-related behavior and impaired spatial learning and memory, lasting for

at least 2 wk after rats had recovered from infection. In concurrence with previous studies, these outcomes indicate that inflammatory events may lead to prolonged alterations in the immune system (5, 44), as well as depression-like behavior and learning and memory impairments in rodents (4, 12, 39). It remains to be determined whether these effects persist, but we speculate that infection accelerates age-associated alterations in immune status and behavior, since similar alterations have been reported in aged individuals. Aging has been associated with a chronic low-grade increase in systemic and central inflammatory markers (16, 35, 52) and a “primed” microglial phenotype displaying certain characteristics of activated microglia (e.g., upregulated number of cytokine receptors and an hypertrophic cell body) and as a heightened reactivity to inflammatory stimuli (16, 31). Moreover, observational studies in humans have related inflammatory events during the life span, such as cytomegalovirus infection, repeated elevations of IL-6, and general infectious burden, with increased pro-inflammatory cytokine production and vulnerability to mental health problems (47, 56, 61, 64).

Influence of infection history on postoperative cognitive impairment and inflammation. In accordance with previous studies, healthy aged rats in the present study showed only a decreased performance in spatial recognition and memory after surgery under general anesthesia, indicating that spatial memory is specifically vulnerable to the effects of surgery in healthy adult rodents (3, 11, 26). The rats showed a robust systemic inflammatory response in the first postoperative days, but 2 wk later cytokine levels in plasma and the brain had returned to control levels. However, we did observe an increased number of microglia and retracted microglial processes in the hippocampus and prefrontal cortex, as well as a decrease in BDNF levels and the number of newly formed neurons in the hippocampus. These findings are in accordance with previous studies, indicating that postoperative neuroinflammation is short lived in healthy rats, but subsequent alterations in BDNF-signaling and hippocampal neurogenesis may be associated with longer lasting postoperative cognitive spatial memory impairment (3, 11, 19, 62).

In contrast, aged rats that experienced an infection before surgery showed not only a decreased performance in location recognition and spatial learning, but also impaired visual recognition and cognitive flexibility. Additionally, we observed signs of decreased interest, as indicated by decreased exploration and increased resting behavior, and increased anxiety-related behavior in these rats (8). These outcomes suggest that prior infection predisposes rats to more severe and generalized cognitive and behavioral impairment after surgery under general anesthesia.

The healthy and infection surgery rats performed similarly on spatial memory tests; the location recognition test and the first MWM probe trial. This may be due to a flooring effect in these tests, since both groups seemed to explore randomly (50% for the location recognition test and 25% for the MWM probe trial) (13, 15). Although this allows no conclusion about the effects of a prior infection on surgery-induced spatial memory impairment, spatial learning in the MWM suggests that spatial task performance may be more severely affected in rats with a preoperative infection.

In the acute postoperative phase, rats that experienced a preoperative infection showed a marginal increase in the in-

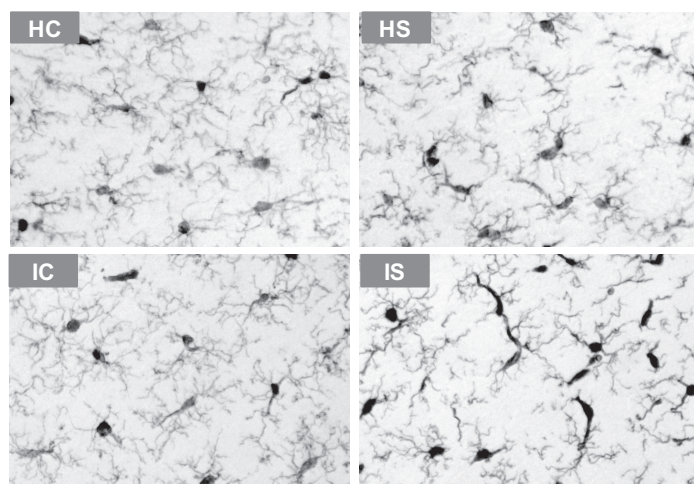
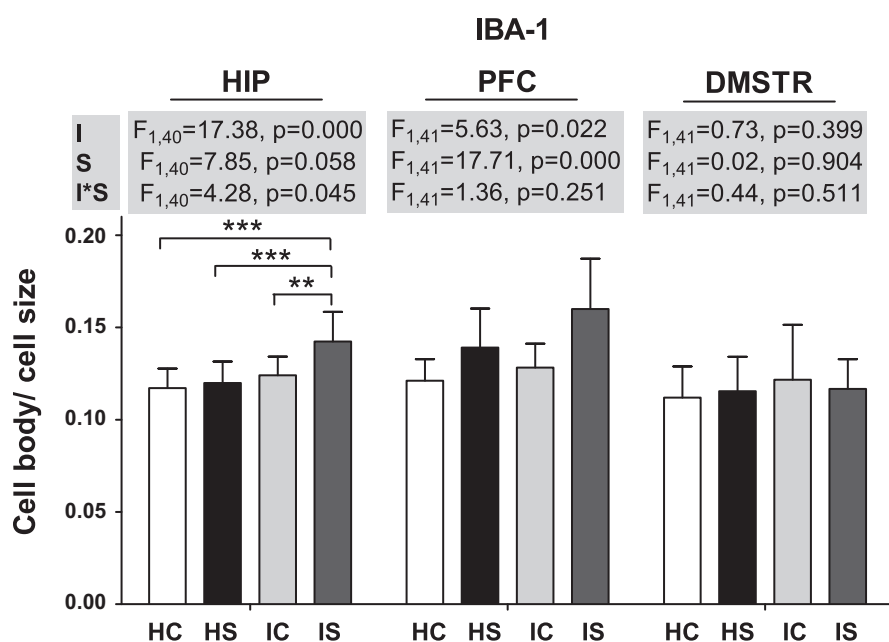


Fig. 6. Microglial morphology. *Top*: representative images of rabbit-anti-ionized-binding adaptor protein (IBA)-1-stained sections of the prefrontal cortex (PFC). *Bottom*: microglia cell body-to-cell size ratio as measure for microglia activation in the HIP, PFC, and dorso-medial striatum (DMSTR) of the brain. ***P* < 0.01, ****P* < 0.001.



flammatory response compared with healthy rats, whereas 2 wk after the surgical intervention, they had significantly increased plasma levels of IL-6 and IL-12. Changes in microglial morphology indicative of microglial activation (elongated cell bodies, a reduction in dendritic processes, and an increased cell body-to-cell size ratio) were observed in the hippocampus and prefrontal cortex. This indicates that a prior infection may predispose rats to more severe and more generalized alterations in neuroimmune functioning after surgery under general anesthesia.

While infection surgery rats displayed changes in microglial morphology in the hippocampus and frontal cortex, cytokine concentrations in these brain regions were unaltered. This might suggest that the microglia of these rats displayed a primed, rather than an activated phenotype (16, 31). In a previous study in young rats, we found that hippocampal and prefrontal microglial activity and cytokine concentrations were only increased in the first postoperative week but not thereafter (26). While in these young healthy rats the microglial morphology had returned to baseline in the second postoperative

week, it is possible that in aged and infected rats the microglia retain a primed phenotype.

It is a topic of debate whether the observed activated microglia are derived from resident microglial cells or are bone marrow-derived macrophages (BMDMs) that are newly recruited to the brain (38, 43). It has been shown that under pathological conditions BMDMs can migrate to the central nervous system and quickly adopt a ramified morphology, indistinguishable from that of microglia (20, 43). Tibia fracture surgery in mice was shown to be associated with infiltration of BMDMs in the brain (59), and a recent study showed that depletion of BMDMs prevents postoperative systemic inflammation, neuroinflammation, and cognitive impairment (14, 59). Interestingly, Nakata et al. (38) showed that the severity of an (ischemic) insult is positively associated with the magnitude of BMDM infiltration. Thus the involvement of microglia and BMDM in the response to surgery may largely depend on the type and severity of the procedure.

To our knowledge, we are the first to investigate the influence of prior infection on postoperative cognitive performance

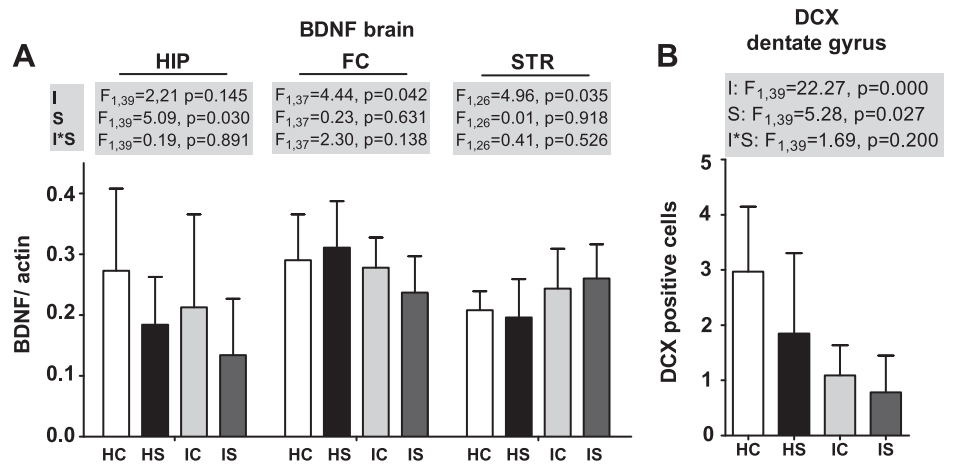


Fig. 7. Markers for neuronal functioning in the HIP, FC, and STR of the brain. **A**: BDNF-to- β -actin ratio. **B**: number of doublecortin-X (DCX)-positive cells in the dentate gyrus.

and (neuro)inflammatory markers in aged rats. However, our outcomes concur with those of other studies showing that a prior infection was associated with an increased neuroinflammatory response and development of cognitive impairment following lipopolysaccharide (LPS) injection (5, 44). Additionally, other authors have reported similar effect in rodents where inflammation was present at the time of surgery. Fildalgo et al. (18) injected mice with a low dose of LPS 2 h before surgery and showed that this led to an exacerbated inflammatory response as well as a more severe impairment of contextual fear memory after surgery. Similarly, metabolic syndrome, which is associated with chronic low-grade inflammation, was shown to exacerbate postoperative (neuro)inflammation as well as postoperative cognitive impairment in both rodent models and clinical studies (17, 28, 29, 57).

As described above, the effects of a prior infection on POCD pathogenesis might possibly be due to changes in immune functioning similar as those seen in aging (24). Alternatively, the observed effects might be the consequence of a two-hit phenomenon where a first hit (the mycoplasma infection) leads to adaptive changes in the organism, leaving it more vulnerable to a second hit (the surgery) (48, 66). For example, it was shown that a loss of intestinal integrity, considered to be an important contributor to excessive inflammation following trauma (21), only occurred after a two-hit of physical injury and infection in rats (49).

Alterations in BDNF signaling as potential mediator of POCD. In a previous study, we hypothesized that the BDNF pathway may potentially act as mediator for postoperative cognitive impairment after neuroinflammation has subsided (26). This hypothesis might explain why, in rats with a prior infection, spatial learning and object recognition were impaired after surgery, while hippocampal and prefrontal levels of inflammatory cytokines were unaltered compared with the nonsurgical control group. In the current study we observed decreased hippocampal levels of BDNF after surgery. Additionally, the BDNF levels in the hippocampus and frontal cortex seem to follow a similar pattern as spatial learning and object recognition, with the lowest outcomes in the infection surgery group. However, because of a large variation in the measured BDNF concentrations, this effect was not significant. In contrast with our hypothesis, rats that had recovered from infection showed increased BDNF concentrations in the striatum,

whereas striatal IL-6 and IL-12 levels were also increased, and cognitive flexibility, presumed to depend on striatal function, was impaired. Previous research indicates that cognitive flexibility may be particularly vulnerable to alterations in cholinergic signaling (23), thus it may be hypothesized that other pathways apart from the BDNF pathway are responsible for the observed changes in cognitive flexibility in our model. However, there are currently no data available to support this hypothesis nor does this hypothesis provide an explanation for the increase in BDNF concentrations in the striatum.

The number of DCX-positive cells in the dentate gyrus was not significantly reduced after surgery in rats with a prior infection. Since in the nonsurgical control rats there was on average less than one DCX-positive cell per section in the dentate gyrus, a further reduction after surgery may be hard to detect.

Limitations. The mycoplasma infection in this study was not intentional. Consequently, it was impossible to control for the severity of the infection in our rats. Moreover, we were unable to directly link specific features of the disease (e.g., duration, bacterial load over time, inflammatory response to the infection, pathophysiological changes/lesions) to the behavioral outcomes or levels of (neuro)inflammatory markers. Therefore, the results should be interpreted very cautiously. However, in our opinion, this situation was a serendipitous event that gave a unique opportunity to study the effects of prior infection on POCD development. *Mycoplasma pulmonis* is one of the most prevalent infectious agents in laboratory rats (34, 51), causing inflammatory airway disease (2, 53). Symptoms include snuffling, sneezing, dyspnea, weight loss, and lethargy (2). Whereas the infection is often clinically silent, unfavorable environmental conditions can enhance the progression and severity of the disease (2, 50). *Mycoplasma pulmonis* has been reported to affect pulmonary cell function and kinetics, reproductive efficiency, arthritis, and the pulmonary and systemic immune system (2). Especially the latter may have influenced the outcomes of our study, and results should therefore be interpreted with caution. However, *Mycoplasma pulmonis* has been frequently used as a model to study respiratory mycoplasmas in humans (9, 53) and as such can be considered to be a clinically relevant pathogen to investigate the influence of prior infection on POCD. To confirm the findings of the

current, observational, study, a carefully controlled inoculation study should be performed.

Although, we have used a standardized symptom scoring method to assess disease severity, it is possible that the symptoms not accurately reflect the degree of infection. Since we did not collect any samples during the preoperative period, it is impossible to determine the degree of infection based on pathophysiological markers nor can we ascertain that the infection was completely resolved in all rats before surgery. However, the baseline IL-6 serum concentrations of the surgery rats show no significant difference. This suggest that in the infected animals systemic inflammation, at least, had resolved before surgery.

In this study we did not include a sham surgery group that only received anesthesia and analgesia. Although, previous studies have not found an effect of anesthesia on POCD development (3, 11, 26, 27), behavioral and neurodegenerative effects of anesthesia have been reported (7, 10). Additionally, prior infection may predispose rats to neurodegenerative effects of anesthesia. Therefore, it cannot be excluded that the observed behavioral impairments were partly caused by the anesthetic agent.

In this study, only 18-mo-old rats were included. Therefore, it is currently impossible to determine whether the outcomes of this study were influenced by the age of the rats.

During this experiment several changes in open-field behavior were observed that are indicative of a reduced interest in the environment and increased anxiety. Therefore, it cannot be excluded that alterations in cognitive task performance are (partly) due to an altered motivational state. However, the distance moved during the MWM probe trials and total object exploration in the novel object and novel location test were not affected by infection or surgery, indicating test outcomes were not solely caused by decreased exploratory activity.

Conclusion. Our findings suggest that in aged rats, infections may be associated with prolonged changes in (neuro)inflammatory signaling and behavior. Additionally, prior infection may predispose rats to more severe and more generalized cognitive and behavioral impairment following surgery under general anesthesia. This seems to fit the hypothesis that exposure to inflammatory events could increase the risk for POCD.

Perspectives and Significance

Recent evidence suggests that an exacerbated inflammatory response in aged individuals may be responsible for the increased risk of POCD with aging. However, while this may explain the increased risk in the aged population in general, it does not fully explain why some older individuals develop POCD and others do not. In the current study we explored the potential influence of infection in the period before surgery on surgery-induced neuroinflammation and cognitive decline in rats. The results indicate that prior infection exacerbates postoperative (neuro)inflammation and cognitive impairment. Carefully controlled studies are necessary to confirm this observation. However, as aged surgical patients often have a history of sickness and infection before surgery, these findings support carefully screening aged surgical patients on infectious status before surgery. In a broader perspective our findings may fit the hypothesis that the sum of inflammatory events during life may determine immune reactivity

and thereby the vulnerability to detrimental side-effects of immune challenges with aging. In humans this may include not only recurrent infections or trauma, but also chronic disease, stress, and life-style choices (e.g., smoking or a high-fat intake). It remains to be determined to what extent these factors predispose patients to POCD. Therefore, future studies should further elucidate on the effect of repeated exposure to inflammatory events during aging with regard to susceptibility to postoperative cognitive impairment.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: I.B.H., B.L.v.L., C.N., E.H., E.A.v.d.Z., and R.G.S. conception and design of research; I.B.H. performed experiments; I.B.H. analyzed data; I.B.H., C.N., E.A.v.d.Z., and R.G.S. interpreted results of experiments; I.B.H. prepared figures; I.B.H. and R.G.S. drafted manuscript; I.B.H., B.L.v.L., E.H., E.A.v.d.Z., and R.G.S. edited and revised manuscript; B.L.v.L., C.N., E.H., E.A.v.d.Z., and R.G.S. approved final version of manuscript.

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