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Short communication

Neutrophil gelatinase-associated lipocalin and microglial activity are associated with distinct postoperative behavioral changes in rats

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HIGHLIGHTS

- We explored the link between postoperative NGAL and behavior in a POCD rat model.
- Plasma and hippocampal NGAL were increased postoperatively.
- Plasma NGAL is associated with postoperative spatial learning impairment.
- Microglial activity is associated with reduced postoperative exploratory behavior.
- NGAL may be a sensitive marker connecting the peripheral inflammatory state to POCD.

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ABSTRACT

Neutrophil gelatinase-associated lipocalin (NGAL) has recently gained interest as a marker for neuroinflammation and associated behavioral dysfunction. We aimed to explore the link between NGAL and behavior in a rat model of postoperative cognitive dysfunction (POCD).

Material collected in two previous studies on POCD was analyzed and associated with outcomes for exploratory behavior and spatial learning. Plasma and hippocampal NGAL and microglial activity were analyzed. Pearson's correlations and backward linear regression were performed to study the associations between behavioral parameters, NGAL concentrations, and microglial activity.

Plasma and hippocampal NGAL were increased following surgery. Plasma NGAL was associated with impaired spatial learning only, microglial activity was associated with exploratory behavior only, while hippocampal NGAL was associated with both behavioral aspects. Spatial learning was best predicted by a model containing plasma NGAL concentrations and hippocampal microglial activity.

NGAL may serve as a sensitive marker in connecting the peripheral inflammatory state to POCD, while postoperative changes in exploratory behavior are better reflected by hippocampal neuroinflammation. These findings warrant further exploration in the role of NGAL in development of postoperative behavioral deficits.

Acute systemic inflammatory events have often been associated with changes in cognition and behavior. A striking example is the observed association between surgery-induced inflammation and postoperative cognitive dysfunction (POCD) in both clinical and rodent studies [1–4]. These studies implicate communication between the periphery and central nervous system, in which systemic inflammation can result in neuroinflammation, influencing neuronal functioning [5].

Neutrophil gelatinase-associated lipocalin (NGAL) has recently gained attention as marker for inflammatory processes associated with behavioral changes [6]. Classically known as biomarker for renal damage, it has become clear that NGAL may reflect inflammatory processes in a variety of tissues, including the brain [7,8]. Circulating levels of NGAL were associated with cognitive and mental dysfunction in chronic inflammatory conditions, including Alzheimer's disease and depression [7–9]. In addition to its

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anti-bacterial properties [10,11], NGAL can cause microgliosis and astrocytosis and sensitize microglia and neurons to pro-inflammatory cytokines and apoptosis [7,12]. A potential role of NGAL in periphery to brain communication was supported by upregulated NGAL levels in the brain following systemic inflammation [10,13]. Thus, NGAL may play a role in the propagation of neuroinflammation and neuronal dysfunction after a systemic inflammatory event.

Recently, we showed increased systemic and central NGAL concentrations after cardiac and abdominal surgery in a rat model for POCD [14]. In another study, we showed that plasma and hippocampal NGAL levels were associated with exploratory behavior in a rat model for heart failure after myocardial infarction [15]. To further explore the potential association of NGAL with postoperative neuroinflammation and behavioral outcomes, we analyzed NGAL in plasma and hippocampal tissue from two of our previous POCD studies. We hypothesized that systemic and hippocampal NGAL levels correlate with microglial activation, spatial memory impairment, and altered exploratory behavior after surgery.

To investigate the association between NGAL concentrations and neuroinflammation with behavioral parameters, we analyzed plasma samples, hippocampal tissue, and behavioral data obtained during two previous studies in male Wistar rats. The first study (experiment 1) compared inflammatory markers and behavior 6 weeks after surgery in healthy young (3 months) and aged (20–21 months) rats (n = 8/group) [16]. The second experiment (experiment 2) compared inflammatory markers and behavior 2 weeks after surgery in aged rats (18 months) that were either healthy or had experienced an infection prior to surgery (n = 12/group) [17]. All experiments were approved by the local animal experiment and welfare committee (DierExperimentenCommissie, Groningen, the Netherlands). For a detailed description of the experimental procedures, we refer to Hovens et al. [16,17]. Briefly, under sevoflurane anesthesia and analgesia (0.01 mg/kg Temgesic), rats received major abdominal surgery including exteriorization of the intestines and clamping of the upper mesenteric artery for 15 (experiment 1) or 30 (experiment 2) minutes. In experiment 2 the rats were additionally equipped with a jugular vein catheter during the surgical procedure. Control animals did not receive surgery or anesthesia.

Behavioral tests were performed in the dark phase, each test was performed once per rat. Open field behavior was recorded for 5 min and time spent on exploration behavior (walking, sniffing and rearing) was used as a measure of interest in the environment [18]. Spatial learning was measured as area under the curve (AUC) for either the percentage of incorrect trials over 9 training sessions in a reward-driven Y-maze spatial learning protocol or the average escape latency over 5 training sessions in the Morris water maze. This yielded one outcome for spatial learning, with increased AUC indicating decreased spatial learning performance.

At sacrifice, blood samples were collected via cardiac puncture, rats were perfused with saline, and brains were collected. Blood was centrifuged and plasma was collected and stored at −80 °C until further analysis. From one hemisphere of each brain the hippocampus was dissected, snap frozen, and stored at −80 °C. Hippocampi were homogenized in a 50 mM Tris-HCL buffer containing 150 mM NaCl, 0.002% Tween-20, and protease inhibitor (Complete Mini, Roche Diagnostics, Indianapolis, USA), sonicated for 5 s twice and centrifuged. Supernatant was collected and diluted to 5 mg/ml protein based on a Bradford assay. NGAL concentrations were determined using the RAT NGAL ELISA kit (BioPorto, Hellerup, Denmark). Plasma samples were diluted 10.000 times and hippocampal supernatant was diluted 10 times in the provided dilution buffer, after which the ELISA analysis was performed according to manufacturer's instructions.

One hemisphere of each brain was emersion fixed with 4% paraformaldehyde for at least 3 days, dehydrated with 30% sucrose, deep frozen, and cut into 30 μm thick sections. Microglia in sections containing the dorsal hippocampus were stained against ionized calcium-binding adapter protein 1 (IBA-1) and morphologically characterized as described previously [19]. Briefly, sections were pretreated with H_2O_2, incubated for 3 days at 4 °C with 1:2500 rabbit-anti-IBA-1 (Wako, Neuss, Germany) in 1% BSA, 0.1% Triton-X at 4 °C, incubated for 1 h with 1:500 goat-anti rabbit secondary antibody (Jackson, Wet Grove, USA) in 1% BSA, incubated for 1 h with avidin-biotin peroxidase complex (Vectastain ABCkit, Vector, Burlingame, USA) and diaminobenzidine labeled. Images were acquired by bright-field microscopy (Leica, 100× magnification) of the dentate gyrus inner blade (DGib), cornu ammonis (CA) 1 and CA3 region, in 3 sections per rat. Using Image Pro Plus software (Media Cybernetics), the total cell body size to total cell size ratio (%) was determined as measure for microglial activity. Microglial activity outcomes of the DGib, CA1, and CA3 were averaged per brain section to yield an average activity outcome for the hippocampus.

For each experiment plasma and hippocampal NGAL concentrations, hippocampal cell body to cell size ratios, and the AUC of the spatial learning paradigm were expressed as percentage of young (experiment 1) or healthy (experiment 2) control rats. Data are displayed as mean ± SEM. Group differences in plasma and hippocampal NGAL concentrations were determined using two-way ANOVA followed by Tukey post-hoc analysis. Pearson's correlation coefficients were determined for experiment 1 and 2 separately and combined. Correlations were determined between 1) microglial activity and NGAL concentrations, 2) spatial learning and exploratory behavior, and 3) these inflammatory parameters and behavioral parameters. Finally, to determine which inflammatory parameters best predicted the behavioral outcomes, backward linear regression was applied with spatial learning and exploratory behavior as the dependent variables and plasma and hippocampal NGAL concentrations and hippocampal microglial activity as independent variables.

Table 1 shows the hippocampal microglial activity and the included behavioral parameters for the experimental groups in study 1 and 2. Fig. 1 displays plasma and hippocampal NGAL concentrations. In experiment 1, there was a significant effect of surgery on plasma NGAL concentrations ($F_{1,32} = 4.54$, $p = 0.042$, Fig. 1A) six weeks after surgery, but no significant effect of age or interaction age*surgery. Similar effects were seen for hippocampal NGAL concentrations ($F_{1,31} = 6.96$, $p = 0.014$, Fig. 1B), as well as a trend for an effect of age ($F_{1,31} = 4.00$ $p = 0.056$). In experiment 2, two weeks after surgery, rats that had experienced an infection had significantly increased plasma NGAL concentrations ($F_{1,32} = 20.89$, $p = 0.000$, Fig. 1C) compared to rats that were healthy, and rats that underwent surgery had significantly increased plasma NGAL concentrations ($F_{1,32} = 7.20$, $p = 0.012$) compared to non-surgical controls. Additionally there was a significant infection*surgery interaction effect ($F_{1,32} = 6.58$, $p = 0.016$); only rats that experienced an infection prior to surgery had significantly increased plasma NGAL concentrations compared to the other groups. Hippocampal NGAL concentrations were significantly affected by infection status ($F_{1,42} = 17.32$, $p = 0.000$, Fig. 1D) only.

When the data of experiment 1 and 2 were combined, there was a significant correlation between plasma and hippocampal NGAL concentrations ($r = 0.489$, $p < 0.001$). Hippocampal ($r = 0.260$, $p = 0.030$) but not plasma NGAL concentrations ($r = 0.034$, $p = 0.790$) were significantly correlated with hippocampal microglial activity. Separate analysis of experiment 1 and 2 showed similar correlations, only the statistical significance of the correlation between hippocampal microglial activity and NGAL was lost. There was no significant correlation between spatial learning performance and exploratory behavior ($r = -0.078$, $p = 0.494$). In the data of experiment 1 and 2 combined, plasma NGAL concentrations were significantly correlated with spatial learning (Fig. 2A) but not