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Inhibition of ERK/CREB signaling contributes to postoperative learning and memory dysfunction in neonatal rats

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Abstract

Exposure to surgery with anesthesia early in life may lead to abnormal behavior, learning and memory in humans. Pre-clinical studies have suggested a critical role of glial cell-derived neurotrophic factor (GDNF) in these effects. We hypothesize that the inhibition of extracellular signal-regulated kinase (ERK)-cAMP response element-binding protein (CREB) pathway contributes to GDNF decrease and the dysfunction of learning and memory. To address this hypothesis, 7-day old Sprague-Dawley male and female rats were subjected to right carotid artery exposure (surgery) under sevoflurane anesthesia. Their learning and memory were tested by Barnes maze and novel object recognition tests started 23 days after the surgery. Blood and brain were harvested at various times after surgery for biochemical analyses. Rats with surgery and anesthesia performed poorly in Barnes maze and novel object recognition tests compared with control rats. Rats with surgery had a decreased GDNF concentration in the brain and urine. The concentrations of urine GDNF were negatively correlated with the performance of rats in a delayed memory phase of Barnes maze test. Surgery increased proinflammatory cytokines in the blood and brain. Intracerebroventricular injection of GDNF attenuated the increased inflammatory response in surgery rats. Surgery inhibited ERK and CREB. Inhibiting ERK reduced GDNF and induced poor performance in Barnes maze and novel object recognition tests of rats without surgery. Surgery also increased brain-derived natriuretic peptide (BNP) in the brain. Intracerebroventricular injection of BNP inhibited ERK and CREB, reduced GDNF and impaired learning and memory. Surgery, ERK inhibition and BNP reduced the expression of synaptic proteins. Our results suggest that surgery increases BNP that inhibits ERK-CREB signaling to

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reduce GDNF, which leads to an unbalanced inflammatory response and a reduced synaptic protein expression for the development of postoperative cognitive dysfunction.

Keywords

extracellular signal-regulated kinase; glial cell-derived neurotrophic factor; neuroinflammation; postoperative cognitive dysfunction; rats

Introduction

A small fraction of patients with surgery and anesthesia early in life may have a risk for abnormal behavior, learning and memory [1]. Similarly, preclinical studies have shown that neonatal animals exposed to surgery and anesthesia may develop abnormal behavior and impairment of learning and memory [reviewed in [2]]. Our previous studies have shown that the decrease of glial cell-derived neurotrophic factor (GDNF) is a key process for these abnormal functions [3,4]. However, it is not clear how GDNF was decreased by surgery. In addition, effective interventions in humans have not been identified to reduce the detrimental effects of surgery. Defining the mechanisms for these effects may help identify interventions to reduce abnormal behavior, learning and memory after surgery.

A previous study has shown that cAMP response element-binding protein (CREB) can regulate GDNF expression in the brain [5]. CREB can be regulated by extracellular signal-regulated kinase (ERK) [6]. Also, stress has been shown to increase brain-derived natriuretic peptide (BNP) [7]. BNP may inhibit ERK [8]. It is not known whether the ERK-CREB signaling pathway plays a role in modulating GDNF in the brain after surgery and whether BNP is altered by the surgery.

It is known that not everyone after surgery will develop abnormal behavior, learning and memory. Thus, it is important if biomarkers can be identified to predict individuals who will have these dysfunctions. However, such biomarkers have not been established. Since the decrease of GDNF plays a critical role in the abnormal behavior after surgery, it is not known whether GDNF levels soon after surgery may have a predicting value for learning and memory dysfunction developed later.

Based on the above information, we hypothesize that surgery increases BNP, which inhibits ERK-CREB signaling to reduce GDNF for learning and memory dysfunction after surgery and that GDNF levels are correlated with memory performance. To address these hypotheses, we subjected 7-day old rats to right carotid artery exposure. This surgical procedure does not affect the motor and major organ functions and has been used in our previous studies [3,4]. Learning and memory were tested.

Materials and Methods

The institutional Animal Care and Use Committee of the University of Virginia (Charlottesville, VA) had approved our animal protocol. The animal experiments were

carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications number 80–23) revised in 2011.

Animal groups

Postnatal day 7 (PND7) male and female Sprague-Dawley rats were littermate-matched and randomly assigned to two groups in the first experiment: (1) control (not being exposed to anesthesia, surgery or any medications), or (2) surgery. These rats were used for learning and memory tests.

In the second experiment, PND7 rats were randomly assigned to: (1) control group, (2) surgery group 1, (3) surgery group 2, or (4) surgery group 3. Rats in surgery group 1, group 2 and group 3 had right carotid artery exposure (surgery) under anesthesia with 3% sevoflurane for 2 h. Their hippocampus was harvested at 1, 2 or 4 days, respectively, for analyzing GDNF, ERK and CREB. In another similar experiment (third experiment), blood and brain were harvested at 2, 4, 8, 24 or 48 h for measuring inflammatory cytokines, complement 3 (C3) or BNP.

In the fourth experiment, PND7 rats were randomly assigned to: (1) control group, (2) surgery group, (3) ERK inhibitor (U0126) group, (4) BNP group, or (5) vehicle group. U0126, an ERK kinase inhibitor [9], BNP or vehicle were given to PND7 rats. The vehicle was 5% dimethyl sulphoxide (DMSO) in water, which was the solvent for U0126. BNP was dissolved in water. Their hippocampus was harvested 48 h after the surgery for biochemical analysis.

In the fifth experiment, PND7 rats were assigned to: (1) control group, (2) surgery group, (3) surgery plus GDNF group, or (4) surgery plus vehicle group. Their brain was harvested 48 h after the surgery for immunostaining and interleukin (IL)-6.

In the sixth experiment, PND7 rats were assigned to: (1) control group, (2) surgery group, (3) U0126 group, or (4) vehicle group. They were subjected to learning and memory tests. Urine was harvested from the control group and surgery group (1, 2 or 4 days after the surgery).

In the seventh experiment, PND7 rats were assigned to: (1) control group, or (2) BNP group. They were subjected to learning and memory tests.

Anesthesia and surgery

The surgery was right carotid artery exposure [3]. Briefly, PND7 rats were anesthetized by 3% sevoflurane. During the procedure, the rat was breathing spontaneously with a facemask supplied with 100% oxygen. Rectal temperature was monitored and maintained at 37°C with the aid of a heating blanket (TCAT-2LV, Physitemp Instruments Inc., Clifton, NJ) during the anesthesia. A 1.5-cm midline neck incision was made after the rat was exposed to sevoflurane for at least 30 min. Soft tissues over the trachea were retracted gently to allow dissection of 1-cm long right common carotid artery free from adjacent tissues with no damage to the vagus nerve. The wound was then irrigated and closed by using surgical suture. The surgery was done under sterile conditions and lasted for about 15 min. After the

surgery, all rats had a subcutaneous injection of 6 mg/kg bupivacaine. Additional medication for postoperative pain control was not needed based on the observation of animal activity and presentation. The total duration of general anesthesia was 2 h. The anesthesia level was maintained to abolish the response to toe pinching.

Intracerebroventricular injection of U0126, BNP or GDNF

Some rats received intracerebroventricular injection of 3 μl 10 mM U0126 (catalogue number: 9903; Cell signaling Technology, Danvers, MA) or 1.2 μg rat BNP (1–32) (catalogue number: RP11121; GenScript Biotechnology, Piscataway, NJ) in 3 μl based on previous studies [10,11]. Others received the injection of 3 μl 5% DMSO in water as the vehicle. Some other rats received intracerebroventricular injection of 0.5 μg recombinant rat GDNF (catalogue number: 512-GF; R&D Systems, Minneapolis, MN) in 3 μl or 3 μl heat-denatured (100°C for 5 min) GDNF as described in previous studies [3,4]. Each rat received only one injection to the right lateral ventricle at PND7. The intracerebroventricular injection was performed as described before [3,4] with the aid of a stereotactic apparatus (SAS-5100, ASI Instruments, Inc., Warren, MI) using the following coordinates: 1 mm posterior to bregma, 1.5 mm lateral from midline and 4.0 mm ventral from the surface of the skull. After the injection, the needle was maintained in place for 1 min to prevent backflow of the injected solution. Animals were anesthetized by sevoflurane for about 5 min to complete the injection.

Collection of urine

Urine samples were collected in the morning and transferred to 1.5 mL plastic tubes. The samples were immediately centrifuged at 3800 rpm for 5 min at room temperature. The supernatant was collected and stored at −80°C until analysis.

Novel object recognition tests

Twenty three days after being exposed to various experimental conditions, rats were subjected to novel object recognition test. As described before [12], two identical objects were placed in opposite sides of the box on the training day. A rat was placed in the center and allowed to explore the box for 10 min. An animal was eliminated from the experiment if the total exploration time on two objects was less than 10 s. Twenty four hours later, a novel object and a familiar object were placed in the same locations as in the training phase. The rat was put in the middle of the box and allowed to explore for 10 min. Animal behavior (the time of exploring novel and familiar objects) was recorded by ANY-maze behavioral tracking software. The ratio of time spent with the novel object to the total exploring time was calculated.

Barnes maze

After novel object recognition test, rats were subjected to Barnes maze test to evaluate their spatial learning and memory as previously described [3,4]. Barnes maze has 20 equally spaced holes in a circular table (SD Instruments, San Diego, CA). Only one hole had a connection to a dark chamber called target box. To begin with, one rat was placed in the middle of the Barnes maze. The rat was encouraged to find and enter the target box by the

application of aversive noise (85 db) and bright light from a 200 W bulb shed on the table. Rats were first trained for 4 days with 3 min per trial, two trials per day, and 15-min interval between trials. The memory test was carried out on day 5 (short-term retention) and day 12 (long-term retention). No test was performed during the period from day 5 to day 12.

Brain tissue and serum harvest

Rats were deeply anesthetized with 5% isoflurane for 2 min and perfused transcardially with saline. Their left hippocampus was dissected out immediately for Western blotting and ELISA and the right cerebral hemisphere from bregma −3 to −6 mm was used for immunostaining later. Briefly, the hippocampus was homogenized on ice in the RIPA buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (catalogue number: 89901; Thermo Scientific, Rockford, IL), and protease inhibitor cocktail (10 mg/ml aproteinin, 5 mg/ml peptastin, 5 mg/ml leupetin, and 1 mM phenylmethanesulfonylfluoride) (Sigma-Aldrich). Homogenates were centrifuged at 13,000×g for 20 min at 4°C. The supernatant was collected and used for Western blotting and ELISA.

The blood samples were allowed to clot at room temperature and were centrifuged at 13,000 g for 20 min. The serum was harvested and stored at −80°C for use later.

ELISA of GDNF in the hippocampus or urine

The amount of GDNF in the hippocampus or urine was determined by using an ELISA kit (catalogue number: BEK-2230, Biosense Laboratories AS, Bergen, Norway) according to the manufacturer's instruction and as described previously [3,4]. The amount of GDNF in each sample was then normalized by its protein content (hippocampal samples) or the volume (urine).

ELISA of IL-6, IL-1β, C3 and BNP in the hippocampus or serum

The amount of cytokines IL-6, C3 and BNP in the plasma and hippocampus was determined by a rat IL-6 Quantikine ELISA Kit (Catalog number: R6000B, R&D Systems, Minneapolis, MN), rat IL-1β ELISA Kit (catalog number: ab100768, Abcam, Cambridge, UK), rat complement 3 ELISA Kit (catalogue number: ab157731, Abcam) and rat BNP 32 ELISA Kit (catalogue number: ab108815, Abcam) according to the manufacturer's instruction.

Chromatin immunoprecipitation assay

The binding of endogenous CREB on *gdnf* gene was evaluated by chromatin immunoprecipitation assay using a kit (Millipore, Burlington, MA) according to the manufacturer's instructions. Cell nuclei were isolated from control rat hippocampus and sonicated to shear the DNA into approximate size at 100 – 500 bp. Chromatin DNA was incubated with 10 μg anti-pCREB antibody (catalogue number: ab15077, Abcam) or rabbit IgG at 4°C overnight. Immunoprecipitated protein-DNA complexes were reversely crosslinked and chromatin-bound DNA was purified using a purification kit (Thermo Fisher Scientific, Waltham, MA). A CREB binding site (CRE) within the rat *gdnf* promoter (Genome Browser, UCSC) was identified by bioinformatics analysis with the gene X plain platform using known DNA-binding motifs described

in the TRANSFAC database. Primer sequences flanking the CRE motif within *gdnf* promoter were as follows: forward: 5'-TTCCAGAGCCTAATACCCT-3' and reverse: 5'-GCTGCCCCTTGATATACAA-3'. Standard PCR reactions using 2 μL immunoprecipitated DNA were performed. PCR products were separated by electrophoresis in 1% agarose gels and visualized by Gel Star™ Nucleic acid gel stain.

Immunofluorescent staining

As we described [13,14], brains were harvested and fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline at 4°C for 24 h, rehydrated and embedded in paraffin. Coronal 4-μm sections of the cerebral hemisphere were cut sequentially and mounted on Superfrost plus microscope slides. Antigen retrieval was performed with Tris/EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0) at 95 – 100°C for 20 min. After being washed in Tris buffered saline (TBS) containing 0.025% triton-X 100, sections were blocked with 5% donkey serum in TBS for 2 h at room temperature and then incubated at 4°C overnight with the rabbit polyclonal anti-ionized calcium binding adapter molecule 1 (Iba-1) antibody (1:200 dilution, catalogue number: 019–19741, Wako Chemicals USA, Richmond, VA). After being rinsed in PBS for three times, the sections were incubated with donkey anti-rabbit IgG antibody conjugated with Alexa Fluor 594 (1:200 dilution, catalogue number: A-21209, Invitrogen, Waltham, MA). After being washed in PBS, sections were counterstained with Hoechst 33342 (Thermo Scientific), rinsed and mounted with Vectashield mounting medium (H-1000; Vector Labs, Burlingame, CA). Images of immunostaining were acquired with a fluorescence microscope equipped with a charge-coupled device camera. A negative control without the incubation with the primary antibody was performed in all experiments. The quantification was performed as described previously [13,14]. Briefly, images of 3 non-overlapping fields in the hippocampal CA1 or dentate gyrus area per section were randomly acquired using a counting frame size of 0.4 mm^2. Three sections per rat were imaged. The number of pixels per image with intensity above a predetermined threshold level was considered as positively stained areas and quantified using the Image J 1.47n software (National Institutes of Health, Bethesda, MD). The immunoreactivity to a protein was quantified by percentage area with positive staining to the total area of the microscopic field. All quantitative analyses were performed in a blinded manner as we did before [13,14]. The final data were normalized by the mean value of animals in the control group and presented in fold changes.

Western blotting

Fifty microgram proteins per lane of various samples were separated on a polyacrylamide gel and then blotted onto a polyvinylidene fluoride membrane. The membranes were blocked with Protein-Free T20 Blocking Buffer (catalogue number: 37573, Thermo Scientific, Logan, UT) and incubated with the following primary antibodies overnight at 4°C: rabbit polyclonal anti-CREB antibody (1:1000 dilution, catalogue number: ab15077; Abcam), rabbit polyclonal anti-p-CREB antibody (1:1000 dilution, catalogue number: ab15077; Abcam), rabbit polyclonal anti-ERK antibody (1:1000 dilution, catalogue number: 9102; Cell signaling Technology, Danvers, MA), rabbit monoclonal anti-p-ERK antibody (1:1000 dilution, catalogue number: 4370; Cell signaling Technology, Danvers, MA), rabbit polyclonal anti-alpha tubulin antibody (1:1000 dilution, catalogue number: 2144; Cell

signaling Technology), rabbit polyclonal anti-GDNF antibody (1:1000 dilution, catalogue number: ab18956; Abcam, Cambridge, MA), rabbit polyclonal anti-postsynaptic density protein 95 (PSD-95) antibody (1:1000 dilution, catalogue number: ab18258; Abcam, Cambridge, MA), or rabbit polyclonal anti-synapsin I antibody (1:1000 dilution, catalogue number: ab64581; Abcam, Cambridge, MA). Appropriate secondary antibodies were used. Protein bands were visualized by Gene snap version 7.08 and quantified by Gene tools version 4.01. The relative protein expression of PSD-95 and GDNF was normalized to that of α-tubulin. The phosphorylated ERK or CREB was normalized by the total ERK or CREB. The results from animals under various experimental conditions then were normalized by the mean values of the corresponding control animals.

Statistical analysis

Results were presented as means ± S.D. ($n \geq 6$) for the data that were in normal distribution or in box plot if the data were not in normal distribution. The data from the training sessions of Barnes maze test within the same group were tested by one-way repeated measures analysis of variance (ANOVA) followed by Tukey's test. The comparison of these training data between groups was performed by two-way repeated measures ANOVA. A two-way ANOVA with rat sex and surgery plus anesthesia as the two factors was performed for the data of memory phase in Barnes maze test. The other data were analyzed by one-way ANOVA followed by Tukey's test or t-test if the data were normally distributed or by one way ANOVA on ranks followed by the Tukey test or rank sum test if the data were not normally distributed. Differences were considered significant at a $p < 0.05$ based on two-tailed hypothesis testing. The relationship between urine GDNF concentrations and learning or memory parameters was examined by linear regression. These statistical analyses were performed with Sigma Stat (Systat Software, Inc., Point Richmond, CA, USA). The value of urine GDNF concentrations in predicting memory deficits was tested by receiver operating characteristic (ROC) analyses. These analyses were performed by MedCalc (MedCalc Software Ltd, Ostend, Belgium).

Results

Surgery under general anesthesia caused learning and memory impairment and reduction of GDNF concentrations

Control rats and rats with surgery and anesthesia required less time to identify the target box with more training sessions in the Barnes maze test (Fig. 1A). Surgery under general anesthesia was not a factor to significantly affect the needed time for rats to identify the target box during the training sessions [$F(1,18) = 0.110$, $P = 0.744$]. However, surgery increased the time for rats to identify the target box 8 days after the training sessions in the Barnes maze test (Fig. 1B). Rats with surgery did not spend as much time as control rats in exploring the novel object (Fig. 1C). These results suggest that rats with surgery have learning and memory dysfunction. To determine whether sex has an effect on the learning and memory, a two-way ANOVA was performed. Sex was not a factor to influence the performance of rats at one or eight days after the training sessions in the Barnes maze test [$F(1,10) = 0.2051$, $P = 0.6603$; $F(1,10) = 0.0846$, $P = 0.7771$, respectively]. However, surgery with anesthesia was a factor to significantly influence the performance of rats at 8

days after the training sessions in the Barnes maze test [F(1,10) = 5.015, P = 0.0491]. There was no interaction between sex and surgery with anesthesia to influence the performance of rats at one or eight days after the training sessions in the Barnes maze test [F(1,5) = 0.0202, P = 0.8926; F(1,5) = 0.0264, P = 0.8774, respectively]. Thus, we did not separate the rats based on sex to analyze the results in the other experiments.

Surgery under general anesthesia decreased the concentrations of GDNF in the hippocampus (Fig. 2A). Since it is not possible to harvest brain tissues from humans for potential translational consideration, urine that is easy to obtain in neonates was harvested to measure GDNF. Similar to the findings of GDNF in the hippocampus, GDNF concentrations in the urine of surgery rats were lower than their preoperative values at 2 and 4 days after the surgery (Fig. 2B). GDNF concentrations in the urine were negatively correlated with the time needed to identify the target box at 8 days after the training sessions in the Barnes maze test whether the urine GDNF concentrations at 4 days after the surgery (Fig. 2C) or 2 days after the surgery ($r^2 = 0.182$, P = 0.033) were considered. To determine whether urine GDNF concentrations have a predicting value, ROC analyses were performed. Urine GDNF concentrations that are lower than 31.5 pg/ml at 4 days after the surgery had a good predicting value for the increase of time (> 2 S.D. increase over the mean value of the group) needed to identify the target box in the Barnes maze [area under the curve (AUC): 0.827] (Fig. 2D). The AUC value was 0.792 when the urine GDNF concentrations at 2 days after the surgery were considered. These results suggest that urine GDNF concentrations early after the surgery may have a predicting value for the learning and memory assessed by Barnes maze test a few weeks after the surgery.

Surgery under general anesthesia caused systemic inflammation and neuroinflammation and GDNF reduced surgery-induced neuroinflammation

Similar to what we have shown previously [15], surgery increased the concentrations of IL-1β and IL-6 in the blood (Figs. 3A and 3B). Surgery under general anesthesia also increased IL-6 and C3 concentrations in the hippocampus (Figs. 3C and 3D). In addition, surgery and anesthesia increased the expression of Iba-1 in the hippocampus. The increase of IL-6 and Iba-1 was inhibited by intracerebroventricular injection of GDNF (Fig. 4). These results suggest that surgery induces systemic inflammation and neuroinflammation and that GDNF can reduce neuroinflammation.

Surgery under general anesthesia increased BNP and decreased ERK-CREB signaling to decrease GDNF

Consistent with findings from ELISA, surgery and anesthesia reduced GDNF in the hippocampus assessed by Western blotting analysis (Fig. 5A). Surgery and anesthesia also decreased the phosphorylated ERK and CREB (Figs. 5B and 5C), the activated fraction of ERK and CREB. Surgery increased BNP in the brain but did not alter BNP concentrations in the blood (Figs. 5D and 5E). To determine the relationship among ERK, CREB and BNP, U0126 and BNP were given intracerebroventricularly. Similar to surgery and anesthesia, U0126, an ERK kinase inhibitor [9], reduced ERK and CREB activation. BNP also reduced ERK and CREB activation (Figs. 6A to 6C). U0126 and BNP reduced GDNF abundance in the hippocampus (Fig. 6D). These results suggest that surgery with anesthesia inhibits

ERK-CREB signaling to reduce GDNF expression and that an event upstream of these effects is BNP increase.

Our previous study has shown that decreased GDNF may be involved in the reduction of spine density after surgery (Xie et al., 2022). Consistent with this finding, surgery and U0126 reduced the expression of PSD95 and synapsin 1, two synaptic proteins. BNP also reduced PSD95 but did not affect synapsin 1 (Figs. 6E and 6F). These results suggest that surgery with anesthesia via the ERK-CREB pathway reduces synaptic protein expression.

Similar to the Western blotting results, U0126 and BNP reduced GDNF concentrations in the hippocampus detected by ELISA (Fig. 7A). A fragment of *gdnf* gene was immunoprecipitated by an anti-phospho-CREB antibody (Fig. 7B), suggesting that CREB may bind *gdnf* gene directly to regulate the expression of GDNF.

ERK inhibition or BNP impaired learning and memory

The time needed for rats to identify the target box was decreased with the increase of training sessions in the Barnes maze test whether control rats, rats with surgery, rats treated with U0126 or vehicle were considered (Fig. 8A). Similar to the animals with surgery, ERK inhibition by U0126 in the brain of rats without surgery increased the time needed for rats to identify the target box one or eight days after the surgery (Fig. 8B). Similarly, rats with surgery and anesthesia or treated with U0126 had a poorer performance than control rats or rats treated with vehicle in the novel object recognition test (Fig. 8C). These results suggest that inhibition of ERK leads to learning and memory dysfunction.

Control rats or rats treated with BNP required less time with more training sessions to identify target box in the Barnes maze test (Fig. 9A). Rats treated with BNP needed a longer time to identify the target box 8 days after the training sessions (Fig. 9B). Rats treated with BNP also spent less time than control rats on the novel object in the novel object recognition test (Fig. 9C). These results suggest that intracerebroventricular injection of BNP impairs learning and memory.

Discussion

Our previous studies have identified an important role of GDNF decrease in the abnormal behavior, learning and memory of the neonatal rats after surgery [3,4]. Consistent with this role, surgery and anesthesia decreased GDNF and induced systemic inflammation and neuroinflammation. GDNF given to the brain attenuated neuroinflammation. In supporting our finding, a previous study has shown that GDNF can reduce inflammatory responses in a cell culture model [16]. This anti-inflammatory effect, along with the knowledge that neuroinflammation is a critical neuropathological process for learning and memory dysfunction after surgery [13,14] and that intracerebroventricular injection of GDNF attenuates abnormal behavior, learning and memory after surgery in our previous studies [3,4,17], suggest that reducing neuroinflammation may be a mechanism for GDNF to attenuate the development of abnormal learning and memory after surgery. This mechanism and our previous findings that intracerebroventricular injection of GDNF can maintain neurogenesis, dendritic arborization and spine density [3,4,17] indicate multifaceted

mechanisms for the reduced GDNF concentrations to contribute to the development of abnormal behavior, learning and memory after surgery. Consistent with those previous studies [3,4,17], surgery and anesthesia reduced GDNF and the expression of synaptic proteins in this study.

How surgery and anesthesia may reduce GDNF expression has not been determined. A previous study has shown that *gdnf* gene has a putative CREB binding site [5]. Since the activity of CREB is regulated by ERK [18], we decided to determine whether the ERK-CREB signaling played a role in the regulation of GDNF expression. In our study, surgery and anesthesia reduced the activated fraction of ERK and CREB. Inhibition of ERK-CREB pathway by U0126 reduced GDNF expression. In addition, CREB protein appeared to bind the *gdnf* gene in the brain. These results suggest that the ERK-CREB signaling inhibition contributes to the decrease of GDNF after surgery. To determine how surgery and anesthesia may inhibit the ERK-CREB signaling, we found that surgery increased BNP. BNP can regulate multiple intracellular signaling molecules including ERK [8,19]. Intracerebroventricular injection of BNP inhibited the ERK-CREB signaling and GDNF expression. These results suggest that BNP increase is an event upstream of ERK-CREB signaling to reduce GDNF after surgery.

To further determine the role of BNP and ERK-CREB signaling in the effects of surgery and anesthesia on the brain, rats without surgery received BNP or U0126 injection into the brain. BNP and U0126 induced learning and memory impairment because these animals had poorer performance in the Barnes maze and novel object recognition tests. These results are consistent with the findings that increase in BNP and ERK-CREB signaling inhibition reduces GDNF concentrations. These results suggest the following event cascade for surgery-induced learning and memory dysfunction: surgery and anesthesia increase BNP, which inhibits ERK-CREB signaling to reduce GDNF that then decreases synaptic protein expression, impairs microstructure of neurons and reduces neurogenesis that lead to learning and memory dysfunction in a delayed phase (Fig. 10). Interestingly, anesthetics, such as volatile anesthetics, can alter the intracellular calcium homeostasis via multiple mechanisms including enhancing calcium release from the endoplasmic reticulum [20]), which can then affect the ERK-CREB signaling [21]. However, it is unclear whether this acute anesthetic effect contributes to the decreased ERK-CREB signaling observed 2 days after surgery and anesthesia in this study.

It is known that postoperative learning and memory dysfunction does not occur to everyone after surgery [22,23]. It is critically important to identify biomarkers for the learning and memory dysfunction after surgery. Our previous clinical trial suggests that the increased concentrations of IL-6 in the blood early during the surgery are associated with postoperative cognitive dysfunction in patients with abdominal surgery [23]. However, harvesting blood from neonates may not be easy. Harvesting urine is practical. Similar to the decrease of GDNF in the hippocampus, GDNF in the urine was decreased. Interestingly, the decrease of GDNF is correlated with the performance of rats in the memory phase of the Barnes maze test and may have a good predicting value for this performance. Thus, decrease of GDNF concentrations in the urine may be a biomarker for learning and memory dysfunction after surgery. This index had a high sensitivity (100%) and a low specificity

(62%) in predicting postoperative cognitive dysfunction. Future studies may investigate how to improve the specificity. One strategy is to combine the urine GDNF concentrations with other potential biomarkers, such as inflammation biomarkers or BNP, for this purpose.

This study has limitations. First, we showed that GDNF in the brain and urine was decreased in rats with surgery. However, we have not determined the source of GDNF in the urine. GDNF is a small protein whose molecular weight is 30 kDa. Surgery can impair blood-brain barrier and may allow GDNF to permeate from the brain to the blood and then to be excreted to the urine. GDNF in the urine may also be produced from cells in the peripheral tissues because cells other than brain cells, such as macrophages, can produce GDNF [24]. Nevertheless, the finding that urine GDNF concentrations may predict the learning and memory function may remain significant in the context that the source of GDNF in the urine is unknown yet. Second, we have not determined how surgery increases the expression of BNP. Stress and inflammation can increase BNP [7,25]. It is possible that neuroinflammation after surgery increases BNP. Thus, a possible vicious cycle may be formed after surgery: surgery induces neuroinflammation that increases BNP, which then inhibits ERK-CREB signaling to reduce GDNF, leading the balance toward proinflammatory status. Future studies are needed to determine whether the increased BNP is due to neuroinflammation caused by surgery.

Conclusions

Our results suggest that surgery and anesthesia increase BNP, which inhibits ERK-CREB signaling to decrease GDNF in the brain. The decrease of GDNF may lead to a proinflammatory status and reduction of synaptic proteins. These effects ultimately result in learning and memory dysfunction after surgery. Our results also suggest a predicting value of urine GDNF concentrations after surgery in the memory performance of these rats. These findings reveal molecular mechanisms for learning and memory dysfunction after surgery and anesthesia.

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Availability of data and materials:

Available upon reasonable request.

Abbreviations:

ANOVA	analysis of variance
BNP	brain-derived natriuretic peptide
C3	complement 3
CRE	CREB binding site

CREB	cAMP response element-binding protein
DMSO	dimethyl sulphoxide
ERK	extracellular signal-regulated kinase
GDNF	glial cell-derived neurotrophic factor
IL	interleukin
PND7	postnatal day 7
ROC	receiver operating characteristic

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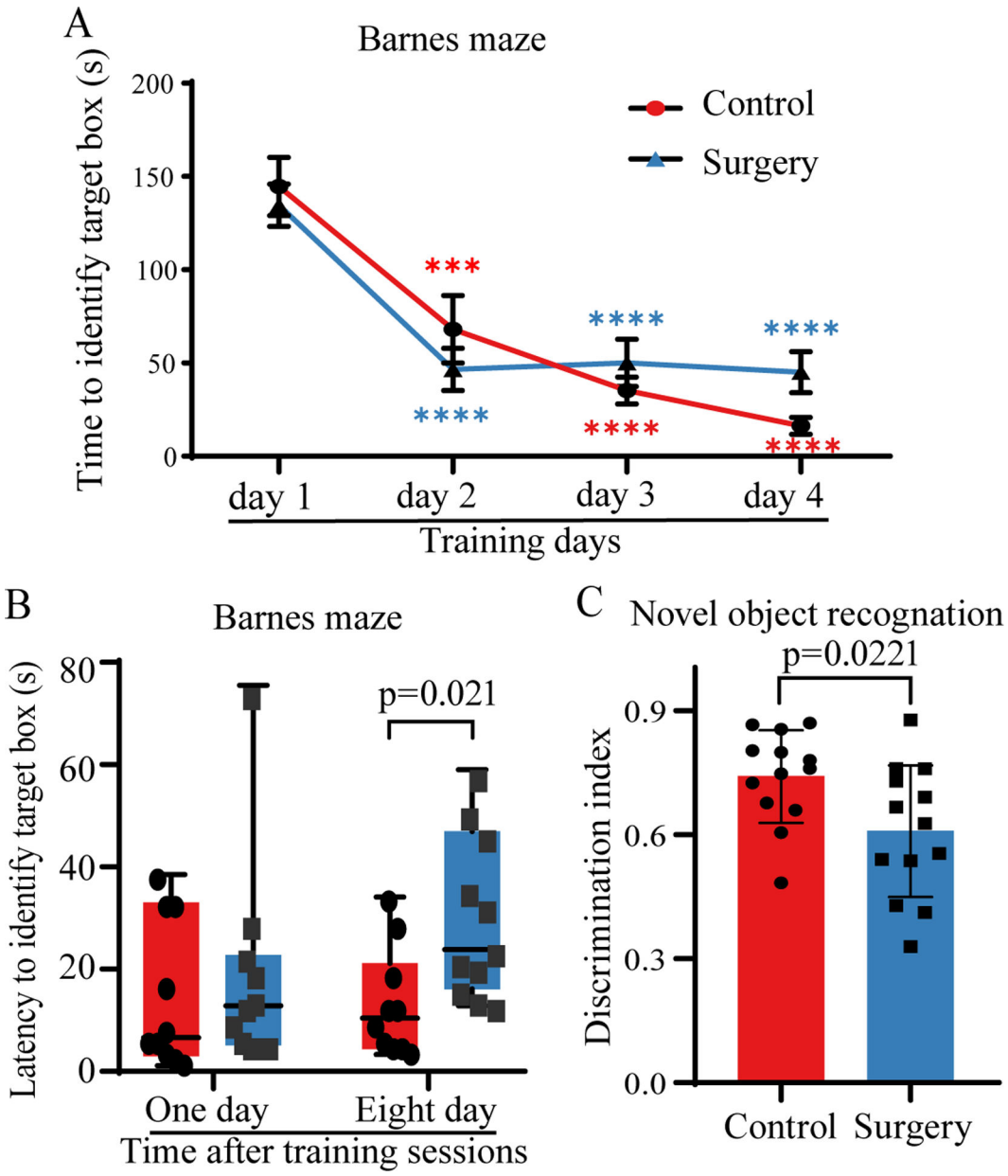

Fig. 1. Surgery induced learning and memory dysfunction.

Postnatal day 7 Sprague-Dawley rats had surgery. These rats and age-matched control rats were subjected to Barnes maze and novel object recognition tests from 23 days after surgery. A: training phase of Barnes maze test. B: memory phase of Barnes maze test. C: novel object recognition test. Results are mean ± S.D. (panels A and C) or in box plot (panel B) (n = 10 for panels A and B, = 13 for panel C). * $P < 0.05$ compared with the corresponding value on day 1.

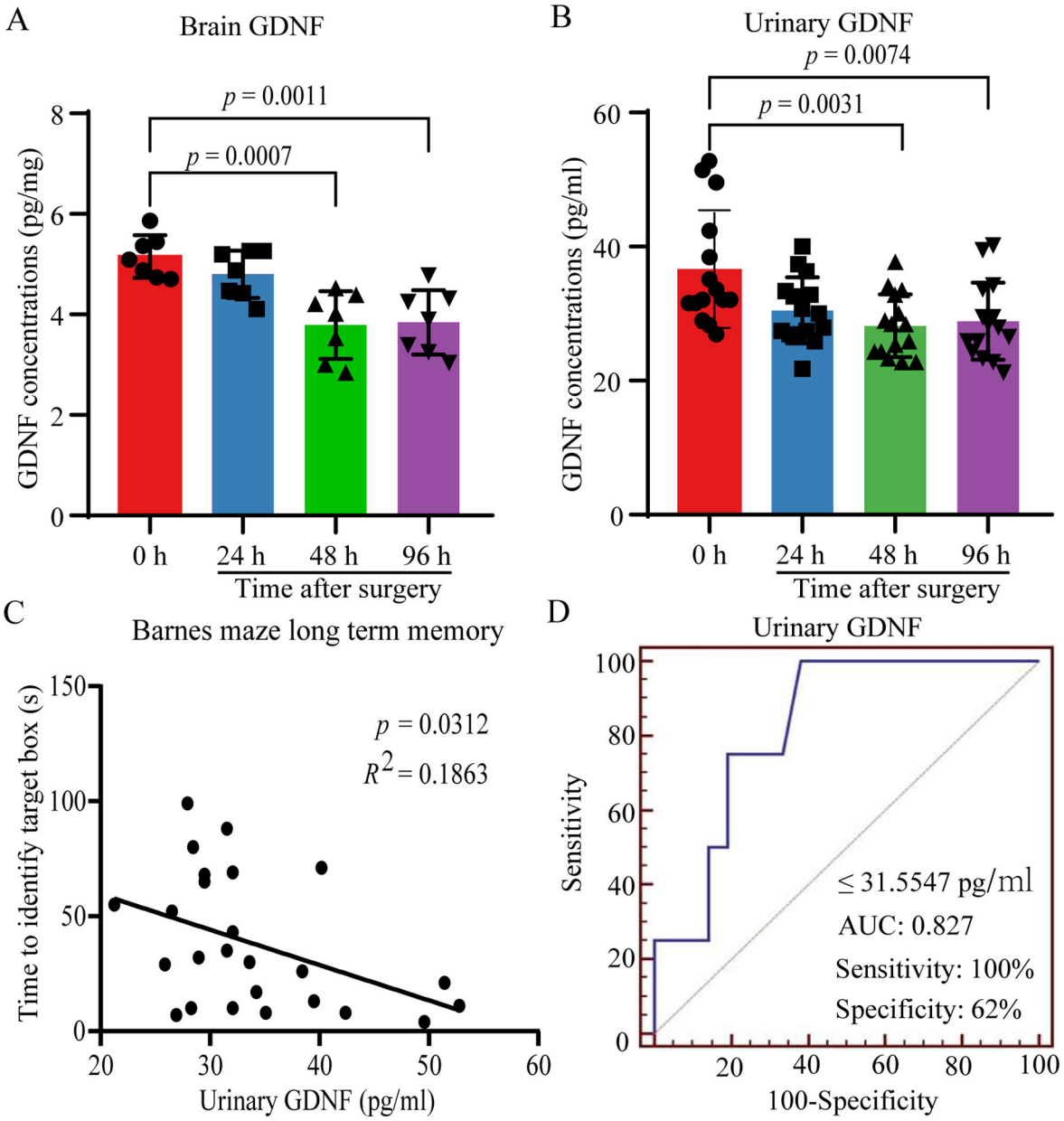

Fig. 2. Surgery decreased GDNF concentrations in the brain and urine.

Postnatal day 7 Sprague-Dawley rats had surgery. Their hippocampus was harvested at various times after surgery in one experiment. The urine was harvested at various times after surgery in another experiment. These rats and age-matched control rats were subjected to Barnes maze test from 23 days after surgery. A: hippocampal GDNF concentrations. B: urinary GDNF concentrations. C: linear regression between urinary GDNF concentrations at 4 days after surgery and latencies to identify the target box 8 days after the training sessions in the Barnes maze test (data of control mice and mice with surgery and anesthesia are included in the analysis). D: ROC analysis of the predicting value of urinary GDNF concentrations at 4 days after surgery for the latencies to identify the target box 8 days after the training sessions to be longer than 2 S.D. above the mean value of the non-surgery

control group (data of control mice and mice with surgery and anesthesia are included in the analysis). Results are mean ± S.D. (panels A and B) (n = 7 for panel A, = 15 for panel B).

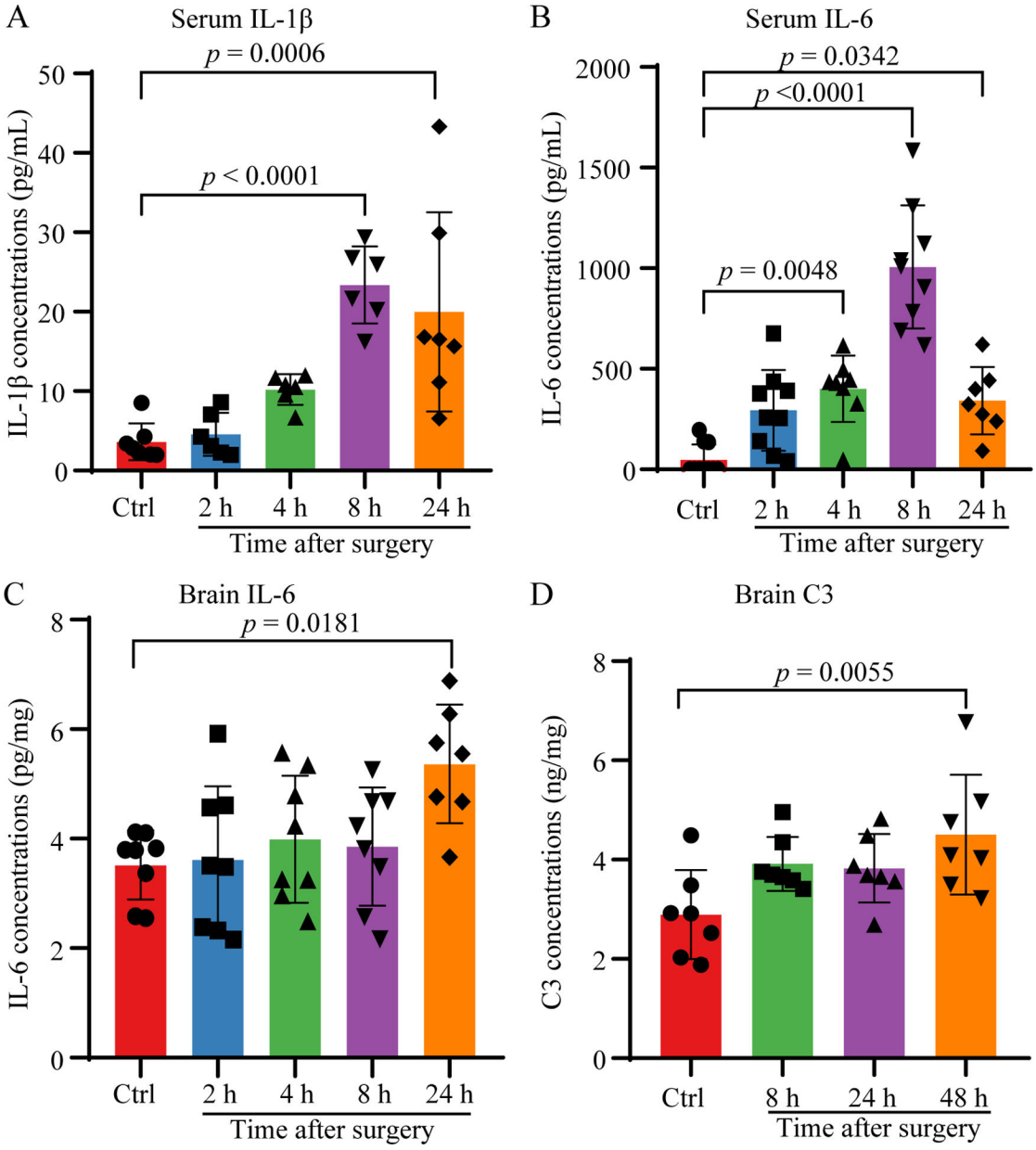

Fig. 3. Surgery increased inflammatory mediators in the brain and blood.

Postnatal day 7 Sprague-Dawley rats had surgery. Their hippocampus and blood were harvested at various times after surgery. A: IL-1β in the blood. B: IL-6 in the blood. C: IL-6 in the hippocampus. D: C3 in the hippocampus. Results are mean ± S.D. (n = 7 – 8). Ctrl: control.

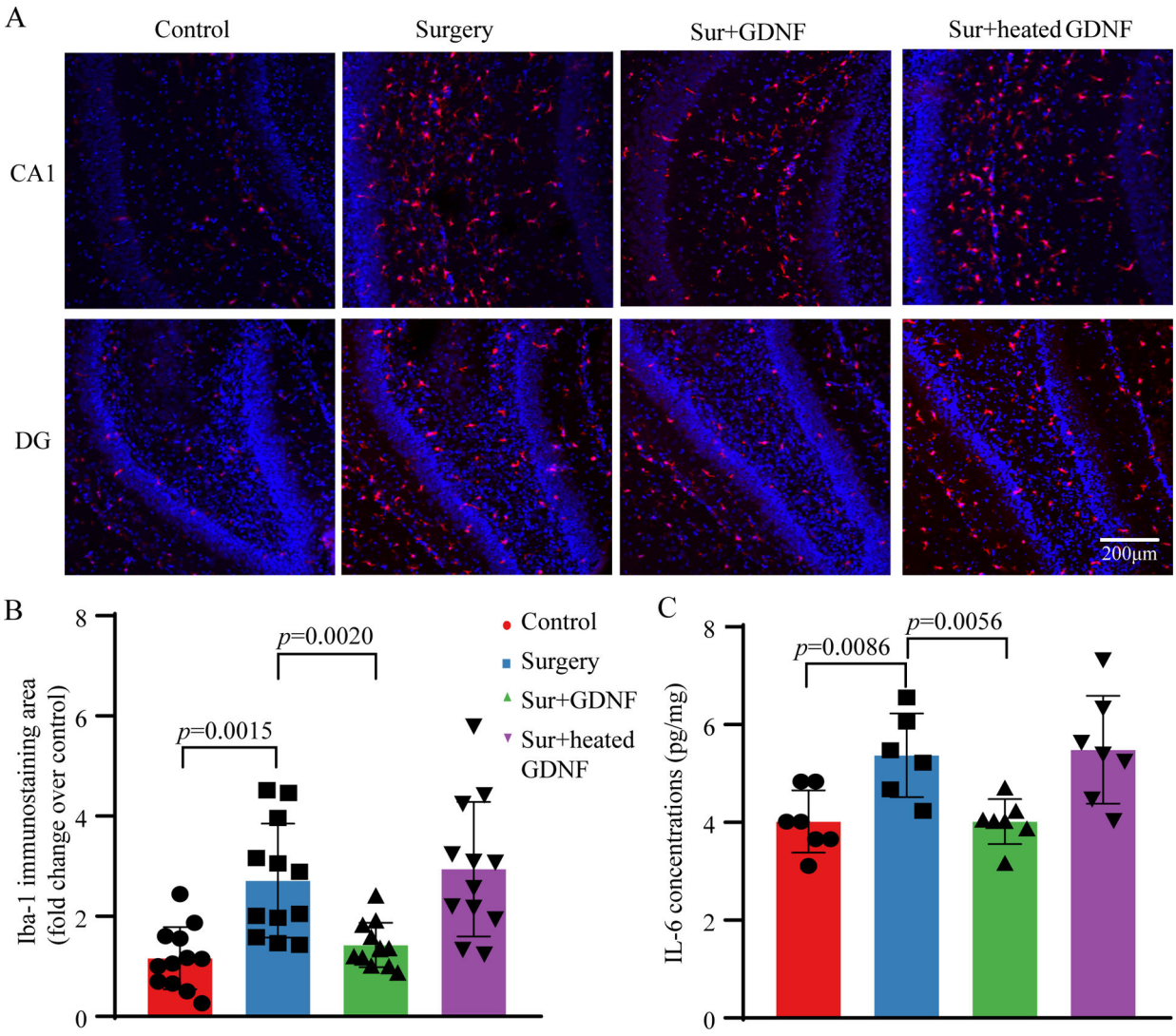

Fig. 4. GDNF inhibited inflammation in the hippocampus.

Postnatal day 7 Sprague-Dawley rats had surgery, surgery plus GDNF or surgery plus heat-denatured GDNF. Their brain was harvested 24 h later for Iba-1 immunostaining or ELISA of IL-6. A: representative immunostaining images for Iba-1. B: quantitative results of Iba-1 staining. C: IL-6 concentrations. Results are mean ± S.D. (n = 12 for panel B, = 7 for panel C). Sur: surgery.

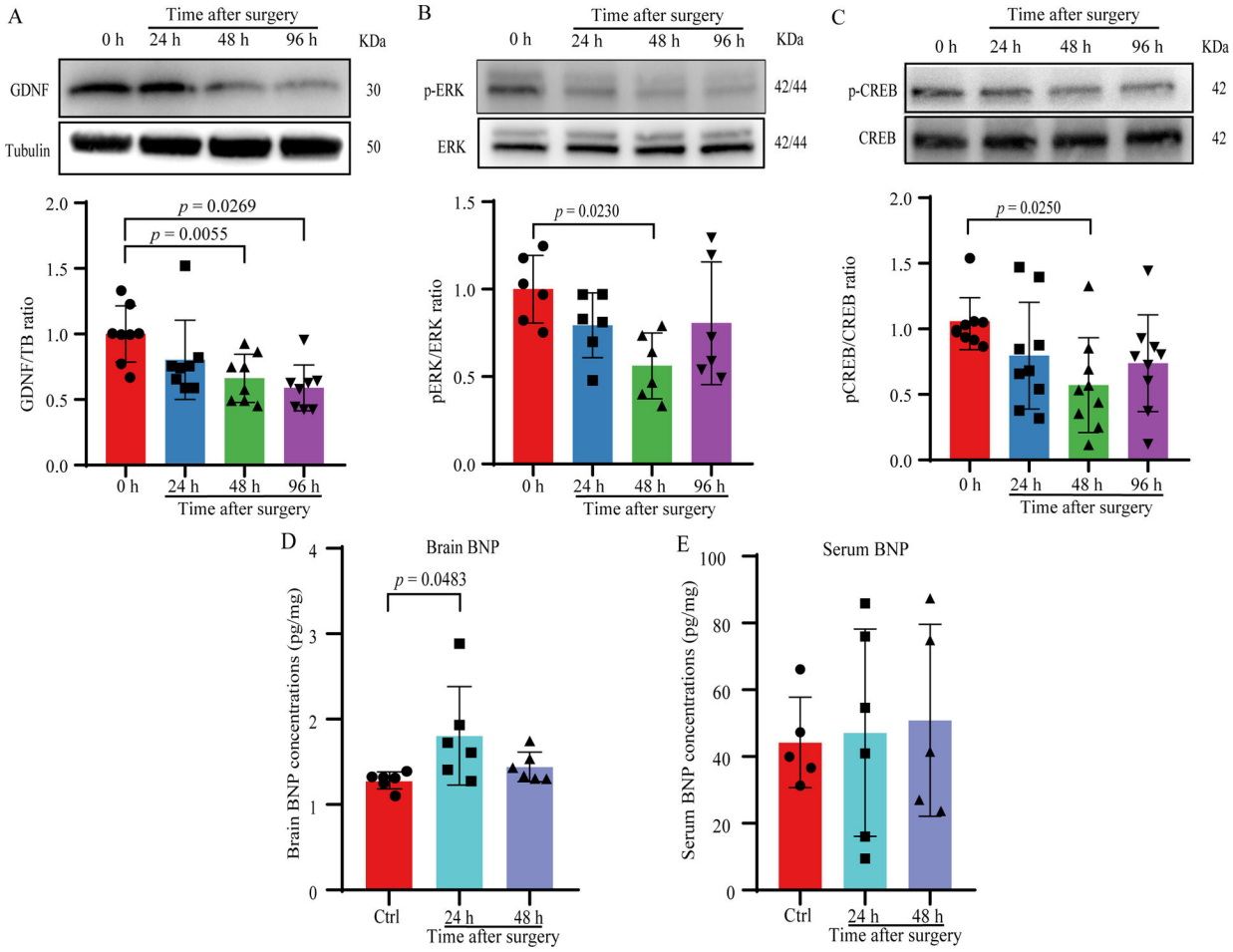

Fig. 5. Surgery reduced GDNF and ERK/CREB signaling and increased BNP in the hippocampus.

Postnatal day 7 Sprague-Dawley rats had surgery. Their hippocampus and blood were harvested at various times after surgery for Western blotting or ELISA. A: GDNF abundance. B: phospho-ERK abundance. C: phospho-CREB abundance. D: BNP concentrations in the hippocampus. E: BNP concentrations in the blood. Results are mean ± S.D. (n = 8 for panels A to C, = 6 for panels D and E). Ctrl: control.

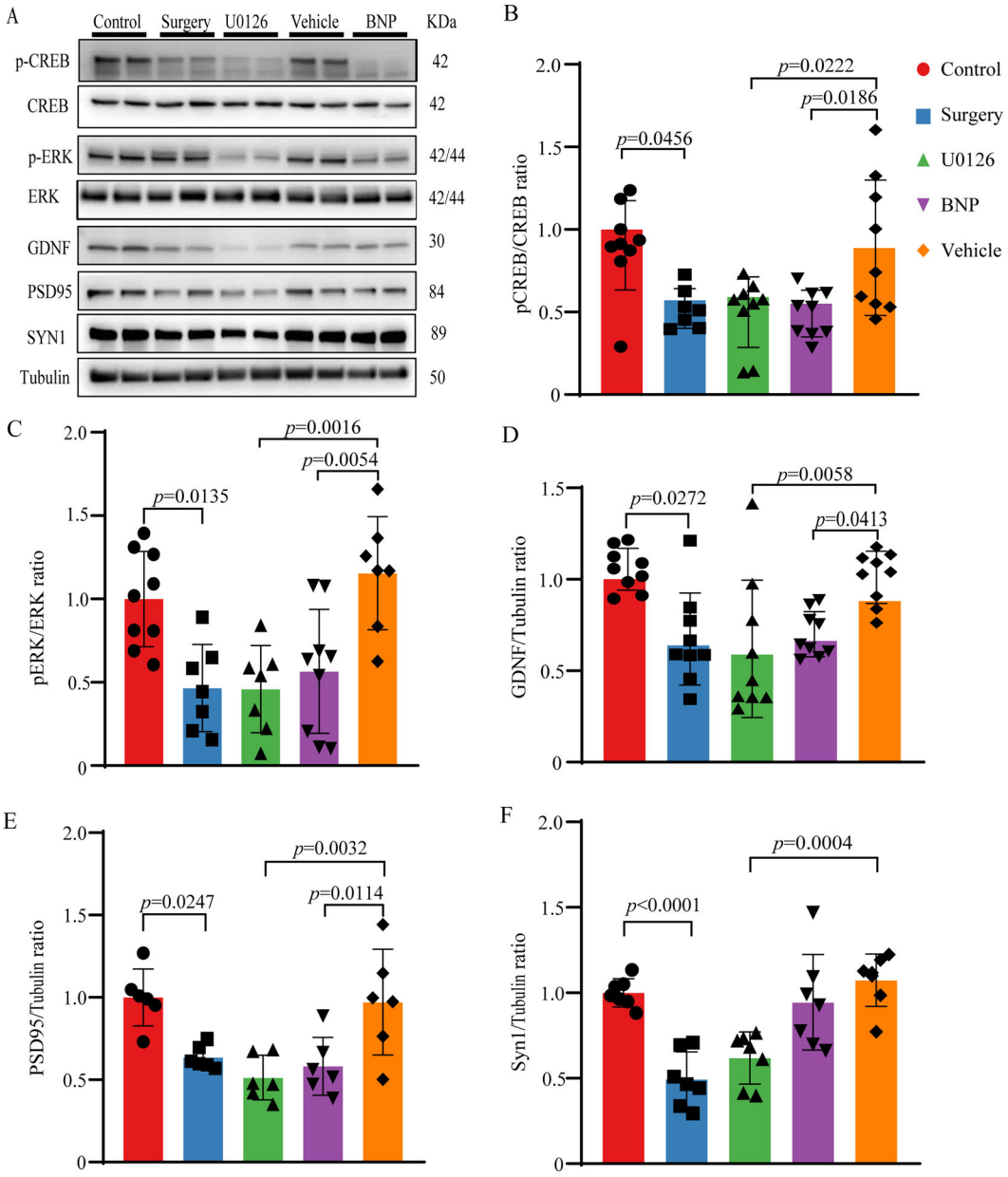

Fig. 6. Surgery, ERK inhibition and BNP reduced GDNF, ERK/CREB activation and synaptic protein in the hippocampus.

Postnatal day 7 Sprague-Dawley rats had surgery or received U0126, BNP or vehicle. Their hippocampus was harvested 48 h later for Western blotting. A: representative western blotting images. B: phospho-CREB abundance. C: phospho-ERK abundance. D: GDNF abundance. E: PSD95 abundance. F: synapsin 1 abundance. Results are mean ± S.D. (n = 7 – 9). Syn1: synapsin 1.

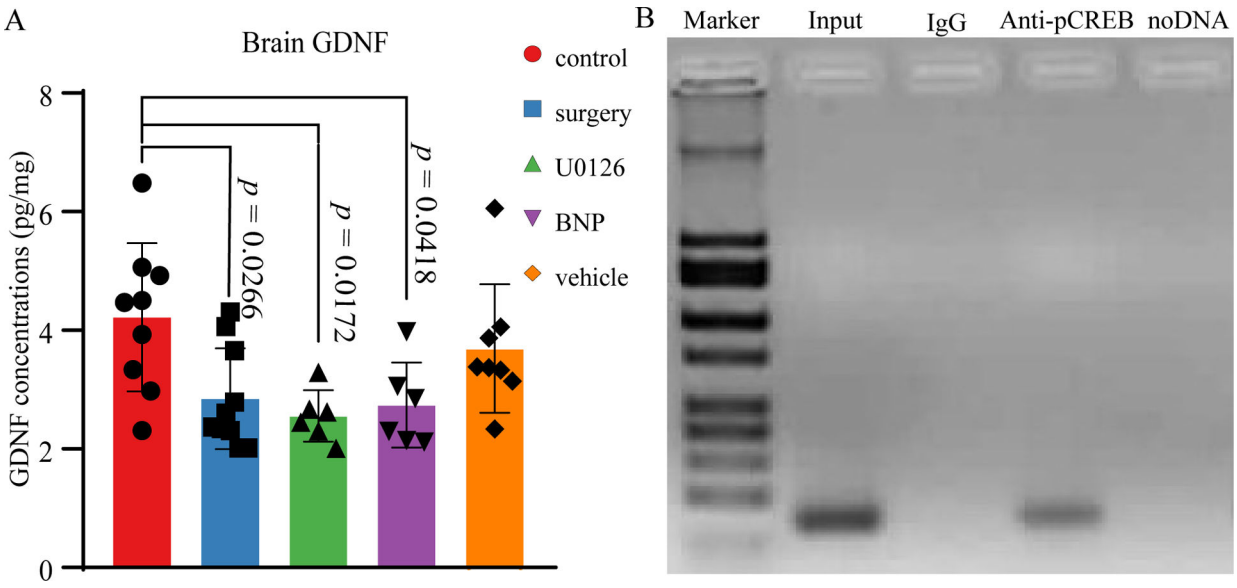

Fig. 7. Surgery, ERK inhibition and BNP reduced GDNF concentrations in the hippocampus.

Postnatal day 7 Sprague-Dawley rats had surgery or received U0126, BNP or vehicle. Their hippocampus was harvested 48 h later. A: GDNF concentrations. B: chromatin immunoprecipitation assay. Results are mean ± S.D. (panels A) (n = 6 – 9). Anti-pCREB: anti-phospho-CREB antibody, noDNA: no DNA sample was loaded.

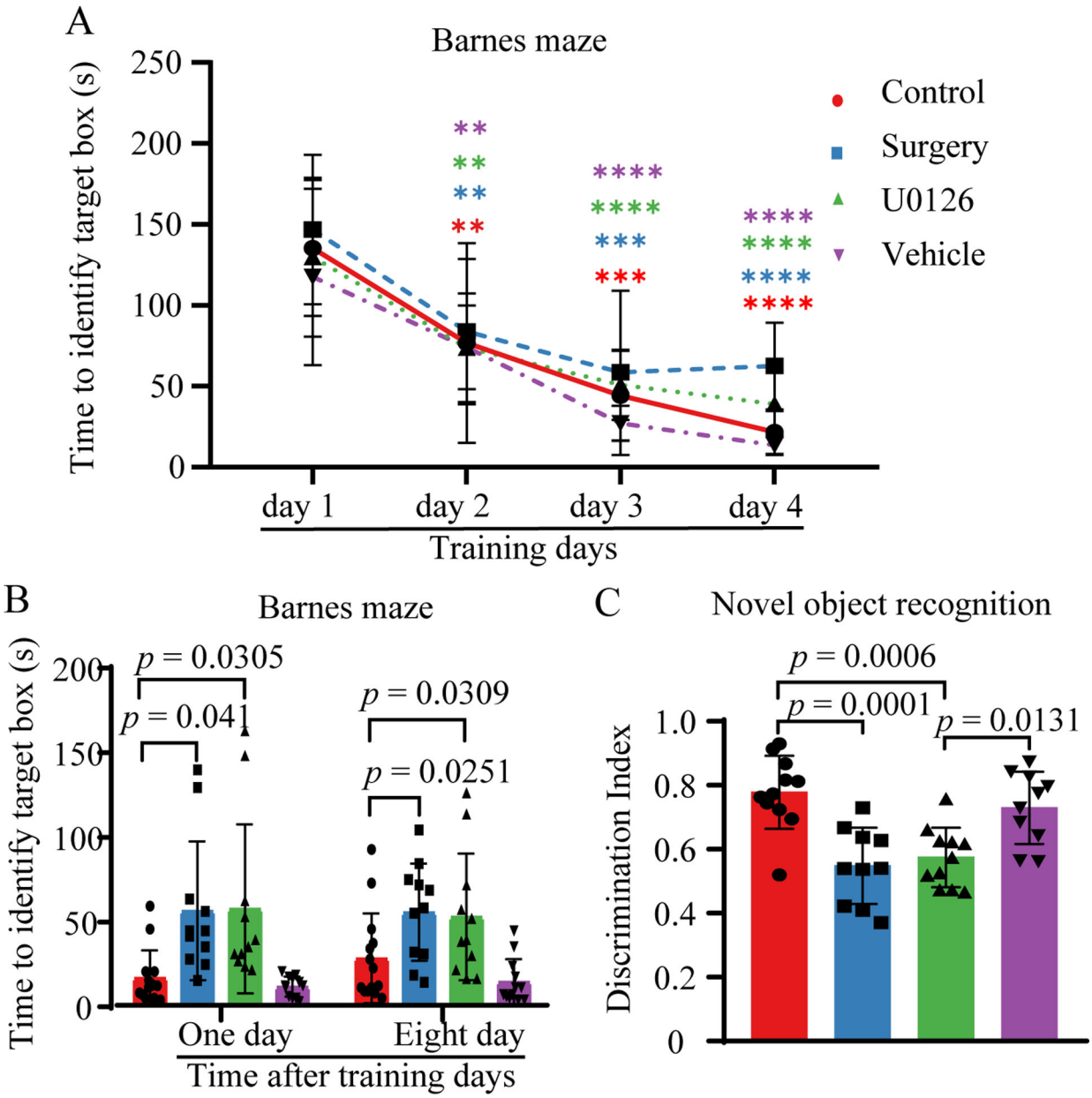

Fig. 8. Surgery and ERK inhibition induced learning and memory dysfunction.

Postnatal day 7 Sprague-Dawley rats had surgery or received U0126 or vehicle. These rats and age-matched control rats were subjected to Barnes maze and novel object recognition tests from 23 days after surgery or receiving U0126 or vehicle injection. A: training phase of Barnes maze test. B: memory phase of Barnes maze test. C: novel object recognition test. Results are mean ± S.D. (n = 10 – 11). * P < 0.05 compared with the corresponding value on day 1.

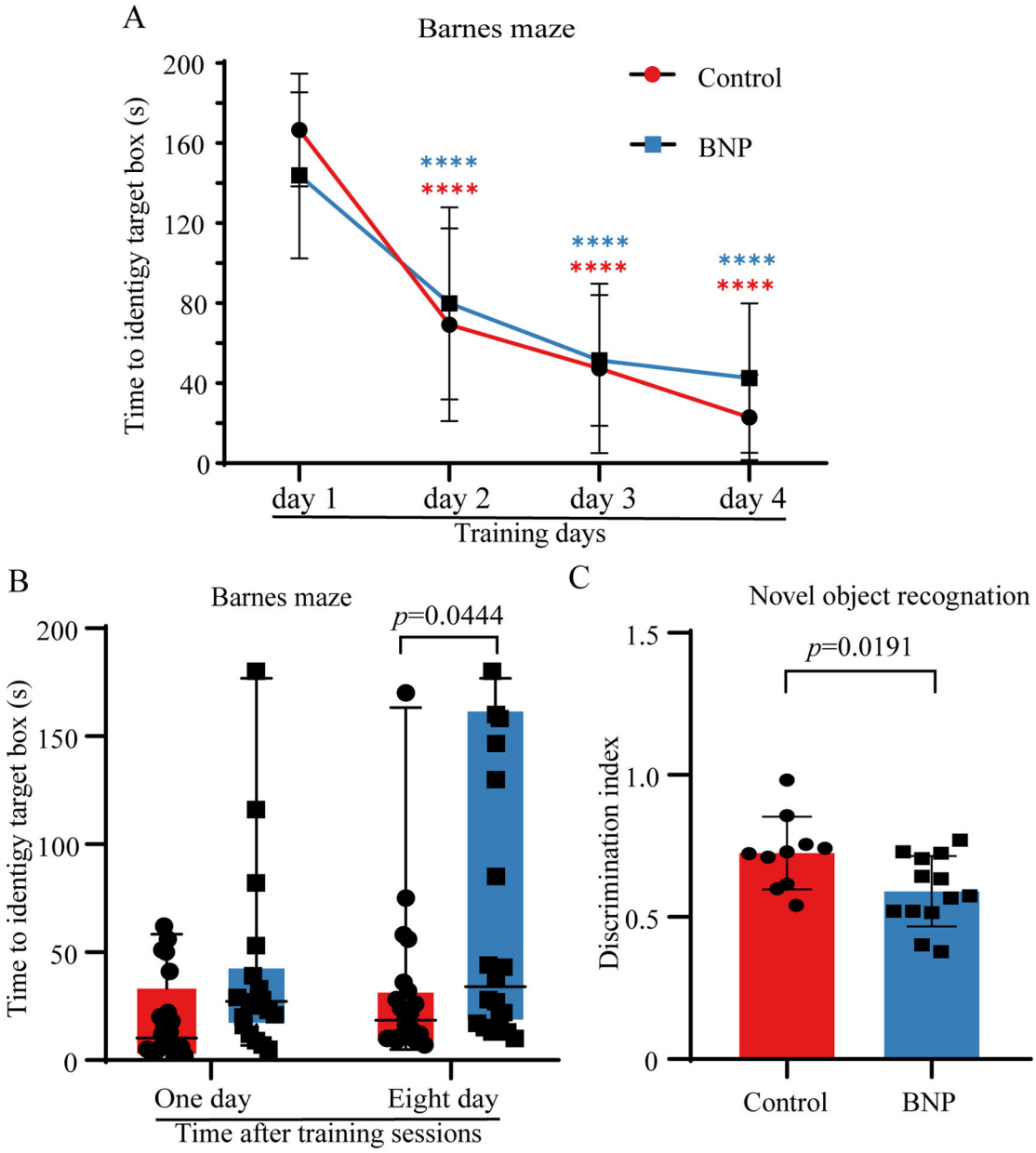

Fig. 9. BNP induced learning and memory dysfunction.

Postnatal day 7 Sprague-Dawley rats received BNP. These rats and age-matched control rats were subjected to Barnes maze and novel object recognition tests from 23 days after surgery. A: training phase of Barnes maze test. B: memory phase of Barnes maze test. C: novel object recognition test. Results are mean ± S.D. (panels A and C) or in box plot (panel B) (n = 10 – 13). * $P < 0.05$ compared with the corresponding value on day 1.

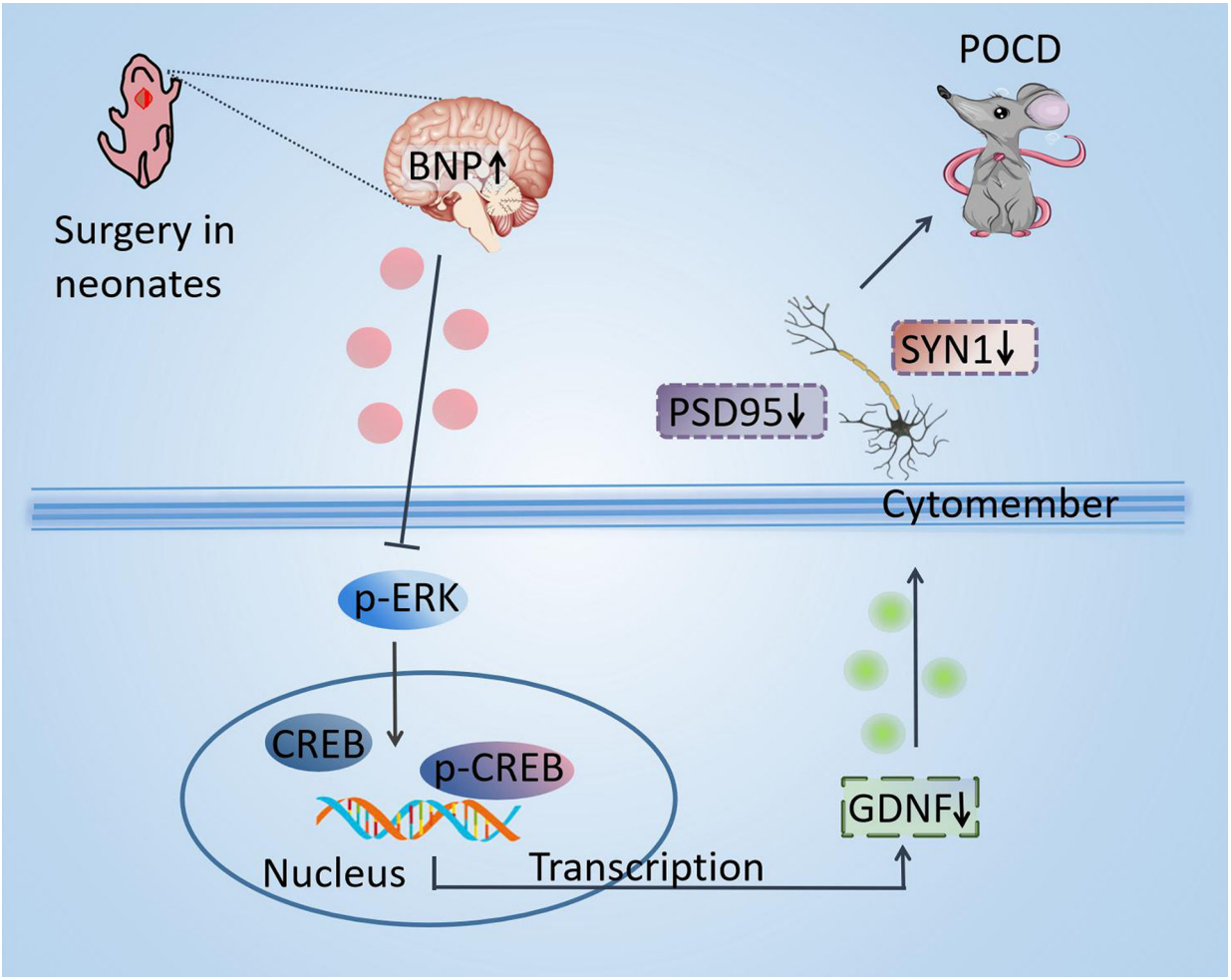

Fig. 10. Diagram of possible signaling pathways identified in this study for surgery-induced learning and memory dysfunction.

POCD: postoperative cognitive dysfunction; Syn1: synapsin 1.