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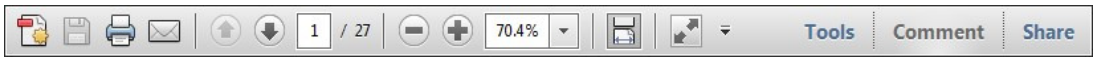

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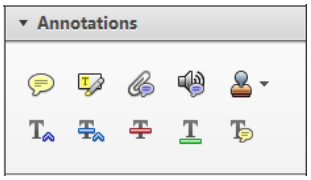

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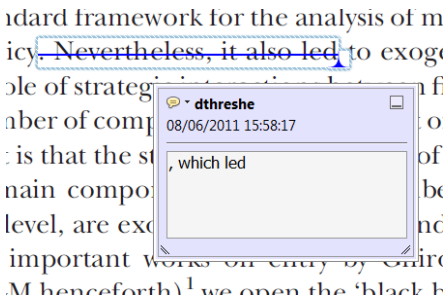

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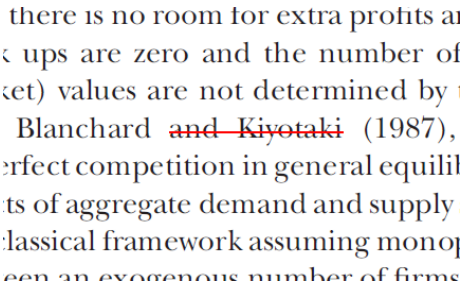

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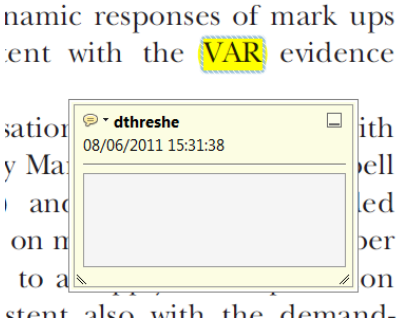

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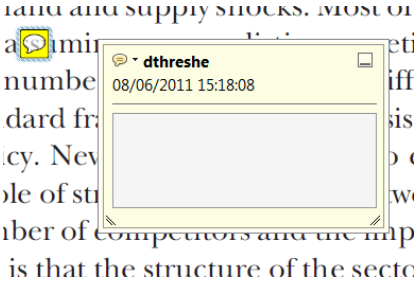

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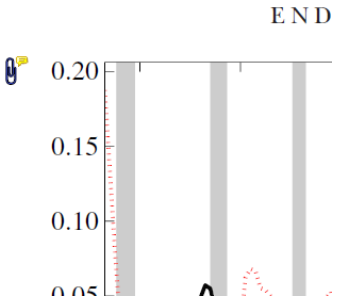

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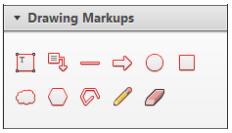

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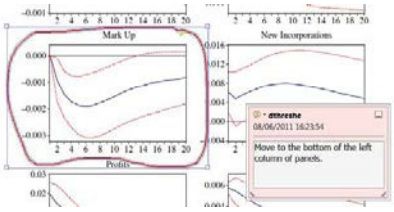

ORIGINAL ARTICLE

CNS Neuroscience & Therapeutics

Surgical Stress Induces Brain-Derived Neurotrophic Factor Reduction and Postoperative Cognitive Dysfunction Via Glucocorticoid Receptor Phosphorylation in Aged Mice

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Aging; Brain-derived neurotrophic factor (BDNF); GR phosphorylation; Postoperative cognitive dysfunction (POCD); Surgical stress.

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SUMMARY

Aims: This study explored whether surgical stress-induced glucocorticoid receptor (GR) phosphorylation is related to postoperative cognitive dysfunction (POCD) in aged individuals. Inhibition of GR activation could be an effective treatment for POCD. **Methods:** A laparotomy was given to C57/BL6 mice in POCD group both 20 and 6 months old. Animals in control group were treated in identical manners except for laparotomy. Cognitive function was evaluated by Morris water maze and elevated plus maze. Western blot and Elisa assay were used to detect related molecules. Mifepristone and roscovitine were treated as inhibitions of GR phosphorylation. **Results:** The cognitive function was impaired, and brain-derived neurotrophic factor (BDNF) was found reduced in aged POCD group. GR translocation into nucleus and elevated GR phosphorylation were found in prefrontal cortex of aged POCD mice. Cyclin-dependent Kinase 5 (CDK5), kinase for GR phosphorylation also elevated in aged POCD mice. With GR antagonist and CDK5 inhibitor, reduction of BDNF and cognitive dysfunction in aged mice were both rescued. **Conclusion:** These results presented a mechanism that surgical stress-induced GR phosphorylation contributes to POCD in aged individuals. Inhibition of GR activation and phosphorylation might be a potential treatment target of POCD.

Introduction

The surgical stress response is intimately associated with adverse outcomes in surgeries of several areas [1]. It has been recognized as one of the risk factors in the pathogenesis of postoperative cognitive dysfunction (POCD) especially in geriatric surgical population [2]. POCD refers to a deficit in cognitive function subsequent to surgery, especially in elder patients [3–5]. Surgery induces various kinds of stress such as surgical impairment, inflammation, ischemia, and psychological stress [6–9]. These stresses contribute to neuronal degeneration associated with hormones disturbance conditions such as aging [10]. During acute or chronic stress, HPA axis (hypothalamic–pituitary–adrenal axis) is activated to release cortisol, which could activate glucocorticoid receptors (GR) leading to cognitive impairment [11–13]. Activation of glucocorticoid receptors includes translocation into nucleus and phosphorylation (primary phosphorylation sites for activation in human: ser203, ser211, and ser226; mice: ser212, ser220, and ser234) following stress controls transcription of genes participating in stress and dementia [14–17] such as BDNF and extracellular-regulated protein kinases (ERK). Patients with POCD presented significantly higher cortisol levels and negatively correlated with Minimal Mental State Examination (MMSE) scores [18,19]. However, whether GR activation participates in surgical stress-induced POCD is not clearly explained.

In this research, we reported that surgical stress-induced BDNF reduction and POCD via activating GR phosphorylation in aged but not in younger mice. GR translocation into nucleus and phosphorylation on ser220 were found in prefrontal cortex of aged mice. The cognitive impairment after surgical stress and following changes might be closely related to GR activation.

Materials and Methods

Animals

Aged (20 months old) and young (6 months old) male C57 mice were bred and kept under standardized housing conditions with

food and water *ad libitum*. All work was approved by the Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch. Both aged and young animals were divided into four groups for different tests: control, POCD, POCD+RU486 (GR antagonist), and POCD+roscovitine (CDK5 inhibitor). As isoflurane inhalation was reported to increase CDK5 [20], we administered an i.p. injection of 5% chloral hydrate for anesthesia and buprenorphine (0.1 mg/kg) for analgesia in all groups. No significant changes on CDK5 with or without chloral hydrate anesthesia (Fig. S2). All of the four groups received chloral hydrate anesthesia. Control group received sterile preparation without surgery as control for possible effects of handling, injection, and anesthesia. POCD group received a laparotomy surgery with saline injection. POCD+RU486 group received surgery with RU486 injection, and POCD+ roscovitine group received surgery with roscovitine.

Surgical Program

Mice were deeply anesthetized by i.p. injection of chloral hydrate (5%) and then received a minor surgery. After the surgical site was shaved and sterilized, a 1.5-cm incision was made in the upper left quadrant through the skin and muscle wall. According to method of Rosczyk HA et al., with minor modification, a sterile probe was then inserted into the body cavity to gently manipulate the internal organs in turn of small intestine, liver, colon, stomach for 1 min. Three dissolvable sutures were used to close the muscle wall and four silk thread sutures were used to close the skin [21]. Before collecting the samples, mice were anesthetized and transported to another room prior to sacrifice.

Drug Exposure

Mice were injected with RU486 (Sigma) dissolved in 30% (wt/vol) propylene glycol + PBS or vehicle acutely after the abdominal surgery as described with minor modification (40 mg/kg, i.p.) [22]. As described with minor modifications [23], roscovitine (Santa Cruz) 10 mg/kg or equal volume vehicle (sterile oil with 0.2% dimethylsulfoxide) was injected through intraperitoneal route daily for 3 days postincision in interaction studies. 6

Physical Signs Analysis

Five extra mice in each treatment groups were prepared to measure the physical signs. 0.3 milliliter of blood was immediately drawn by cardiac puncture. Blood gas testing was carried out immediately using a handheld i-STAT analyser (Abbott Point of Care Inc., Princeton, NJ, USA). The animals were then euthanized and not used for any other part of the study.

Morris Water Maze (MWM)

On day 5 after surgery (3 days for recovery from surgery and 1 day for adaption of experimental environment), mice were used to complete Morris water maze test. As previously described [24] with minor modifications, all groups of 8 mice each were tested for memory using MWM test by investigators blinded to the group conditions. Briefly, the animals received four training trials daily for seven consecutive days. During each trial, the mice were placed gently in the water facing the wall of the maze at one of the four equally spaced start positions. The time to locate the submerged platform (defined as the latency cutoff time of 60 seconds) and swim velocity were recorded. On test day 8, a series of probe trials were conducted, whereby the platform was removed. The percentage of time spent in the previous target platform quadrant (IV) in 60-s period was determined.

Elevated Plus Maze (EPM)

On day 5 after surgery, another groups of mice (n = 8 for each group) were used to complete the elevated plus maze. The protocol of elevated plus maze was as previously described [25] with minor modifications. Elevated plus maze test consists of four polycarbonate arms extending 11.5 inches in length, two of which have walls 6.5 inches in height. The plus maze was elevated from the floor at the height of 24 inches. Mice were placed in an open square dividing the four arms of the chamber with an overhead camera recording the time spent on the open arms of the plus maze during each 5-min test session.

Preparation of Nuclear and Cytoplasmic Extracts

Tissue of mice in all groups was prepared after the elevated plus maze tests on day 7 after surgery. Preparation of nuclear and cytoplasmic extracts was carried out as previously described [26]. To prepare cytoplasmic and nuclear extracts, tissue homogenates were allowed to swell by adding 0.4 μL/μg of cold lysis buffer (10 mM HEPES, 10 mM KCl,0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl -fluoride [PMSF, pH 7.9]) with protease and phosphatase inhibitors (0.2 M NaCl, 0.1 M HEPES, 10% glycerol, 2 mM NaF, 2 mM Na4P2O7, 4 U/mL aprotonin, 2 mM DTT, 1 mM EGTA, 1 μM microcystin, and 1 mM benzamidine). The removed cytoplasmic fraction was stored at −20°C. The pellets containing crude nuclei were resuspended in 50-μL extraction buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM PMSF, pH 7.9), incubated on ice for 30 min, and centrifuged at 12,800 g for 10 min to obtain the supernatant containing nuclear extracts. Another two groups of mice were killed and took samples on day 1 and 13 after surgery.

Western Blot

Western blot analysis was used to determine the expression of related proteins as described [26] with the following modifications. Briefly, sixty micrograms of protein per lane was loaded on 10% SDS-PAGE for protein in nuclear and forty micrograms for protein in cytosol. After transferred onto PVDF membrane, the following primary antibodies were used: sheep anti-BDNF antibody (1:5000, millipore); rabbit anti-GR and anti-p-GRs220 antibody (1:12000, CST); rabbit anti-CDK5 antibody (1:5000, BD); rabbit anti-p35 antibody (1:5000, BD); rabbit anti-p-GRs212 (1:2000, Anbo); and rabbit anti-p-GRs234 (1:12000, CST). 7 8,9 10

Immunohistochemistry and Confocal Microscopy

Tissue preparation and immunohistochemistry were performed as described (Xian et al., 2009) with minor modifications.

Free-floating sections (18 μm thick) were processed for free-floating immunohistochemistry. The primary antibody was applied overnight at 4°C. Secondary antibody used was Alexa Fluor 488 labeled-goat anti-rabbit IgG (MicroProbe, 1:2000). Nuclei were counterstained with Hoechst 33258 (Invitrogen, Carlsbad, CA, USA). Fluorescence images were acquired with a confocal laser scanning microscope (LSM510; Carl Zeiss Co., Oberkochen, Germany) using a 505-nm-long path filter for emission and an argon ion laser for excitation (488 nm). No fluorescence was detected with the primary antibody omitted.

Elisa Kit Assays

Plasma cortisol level was detected with ELISA kit (Enzo, ADI-900-071) according to the procedures provided by the manufacturer. Plasma supernatants were collected on day 1, 7, and 13 postoperation and stored at −80°C until analysis. IL-1β, IL-6, and TNFα was measured in the protein from prefrontal cortex of mice, using a commercially available ELISA kit according to the procedures provided by the manufacturer (IL-1β and TNFα from Abcam; IL-6 from Pierce). The supernatants were collected on day 7 after surgery and stored at −80°C until assays were performed.

Statistical Analysis

Statistics were calculated using functions provided in GraphPad Prism 5 for Windows (GraphPad Software Inc., La Jolla, CA, USA). All data in the text and figures were presented at least three independent experiments and were expressed as mean ± standard error (SEM). Data in escape latency trails of MWM were assessed with two-way ANOVA (treatment condition × day) as described [27] with a minor modification. One-way ANOVA followed by Tukey's *post hoc* tests was performed to compare data in probe trail and other data among multiple groups [27]. Two-tail unpaired *t*-test was performed to compare the data between two groups. The correlation analysis was completed with function of GraphPad Prism 5. Mean values were considered significantly different at $P < 0.05$.

Results

Surgical Stress Induces Postoperative Cognitive Dysfunction in Aged but not in Younger Mice

We used Morris water maze and elevated plus maze to detect the effects of surgical stress on cognition and emotion in 20 (Figure 1A) or 6-month-old (Figure 1B) mice. There was a significant difference on escape latency in aged mouse (two-way ANOVA, treatment condition × day, $F(1,98) = 19.06$, $P < 0.001$ for treatment condition) but not the younger mouse ($F(1,98) = 0.2388$, $P = 0.6261$ for treatment condition). We found the POCD group performed longer latency to reach the hidden platform on day 4, 5, 6, 7 ($P < 0.05$), and shorter time spent in target area (Q IV) with a removed platform ($P = 0.0129$) (Figure 1A) compared with 20-month-old control group in MWM tests. No significant difference was found in visible trail and crossing numbers (Fig. S1). The swimming speed had no difference between the two groups ($P = 0.5595$) (Fig. S6). We also found that 20-month-old POCD group spent shorter time in the open arm on elevated plus maze test ($P = 0.0179$) (Figure 1A). However, in 6-month-old group, no significant difference was recorded between POCD and control in both MWM (escape latency, $F(1,98) = 0.2388$, $P = 0.6261$ for treatment condition; probe time, $P = 0.2208$) and EPM tests ($P = 0.2133$) (Figure 1B). BDNF is a biomarker of neuron survival and cognition [28,29], and it was involved in POCD [30]. We detected BDNF level in prefrontal cortex of 20-month-old POCD and control mice on day 7 postoperation (Figure 1C). BDNF had an appreciable reduction in POCD group compared with control group (two-tail unpaired *t*-test, $P < 0.001$). We also compared BDNF among 4 groups with a two-way ANOVA (treatment × age) analysis (Fig. S3). The result turns out to be age but not treatment factor had a significant effect on BDNF expression. The level of BDNF was increased in aged mice and was significantly increased while surgery along with aging (Fig. S3). There is no significant changes in 6-month-old mice (two-tailed unpaired *t*-test, $P = 0.7660$). These results indicated that surgical stress could induce the cognitive dysfunction in aged mice rather than in young.

Surgical Stress Elevated Glucocorticoid Receptor Function in Nucleus in Prefrontal Cortex of POCD Mice

To demonstrate whether surgical stress could induce GR activation, translocation into nucleus and phosphorylation, we prepared nuclear and cytoplasmic extracts of prefrontal cortex of POCD and control mice on day 7 postoperation. The level of GR in nucleus was found elevated in 20-month-old POCD group compared with control group ($P = 0.0253$) (Figure 2A & B). Oppositely, GR level had a slight decrease in cytosol ($P = 0.0331$) (Figure 2A & B). The rate of GR level in nucleus/cytosol had a significant difference ($P = 0.0257$) (Figure 2A & B). No significant change was found on GR translocation in 6-month-old group ($P > 0.05$) (Figure 2C & D). Results are similar in immunofluorescence assay (Fig. S4). Increased GR translocation into nucleus was found in POCD group rather than control. These data suggested that surgical stress induced GR translocation into nucleus in elder but not in younger mice.

Surgical Stress Elevated GR Phosphorylation at ser220 by CDK5 and its Regulator in Prefrontal Cortex of POCD Mice

While activated by ligands, GR transferred into nucleus and then phosphorylated. The phosphorylation of GR was detected in POCD and control mice on day 7 postoperation. Ser220, one of the most principal phosphorylation sites of mice in nucleus for GR activation, was found elevated in 20-month-old POCD mice ($P = 0.0136$) (Figure 3A). Other sites were also considered such as ser212 ($P = 0.2303$) and ser234 ($P = 0.3860$), without significant changes, however (Figure 3A). We also compared pGRs220 among four groups with a two-way ANOVA (treatment x age) analysis (Fig. S3). Age but not treatment factor had a significant effect on pGRs220 expression. The level of pGRs220 was increased in aged mice and was significantly increased while surgery along with aging (Fig. S3). CDK5 is an important kinase for GR phosphorylation. We use Western blot to detect whether CDK5

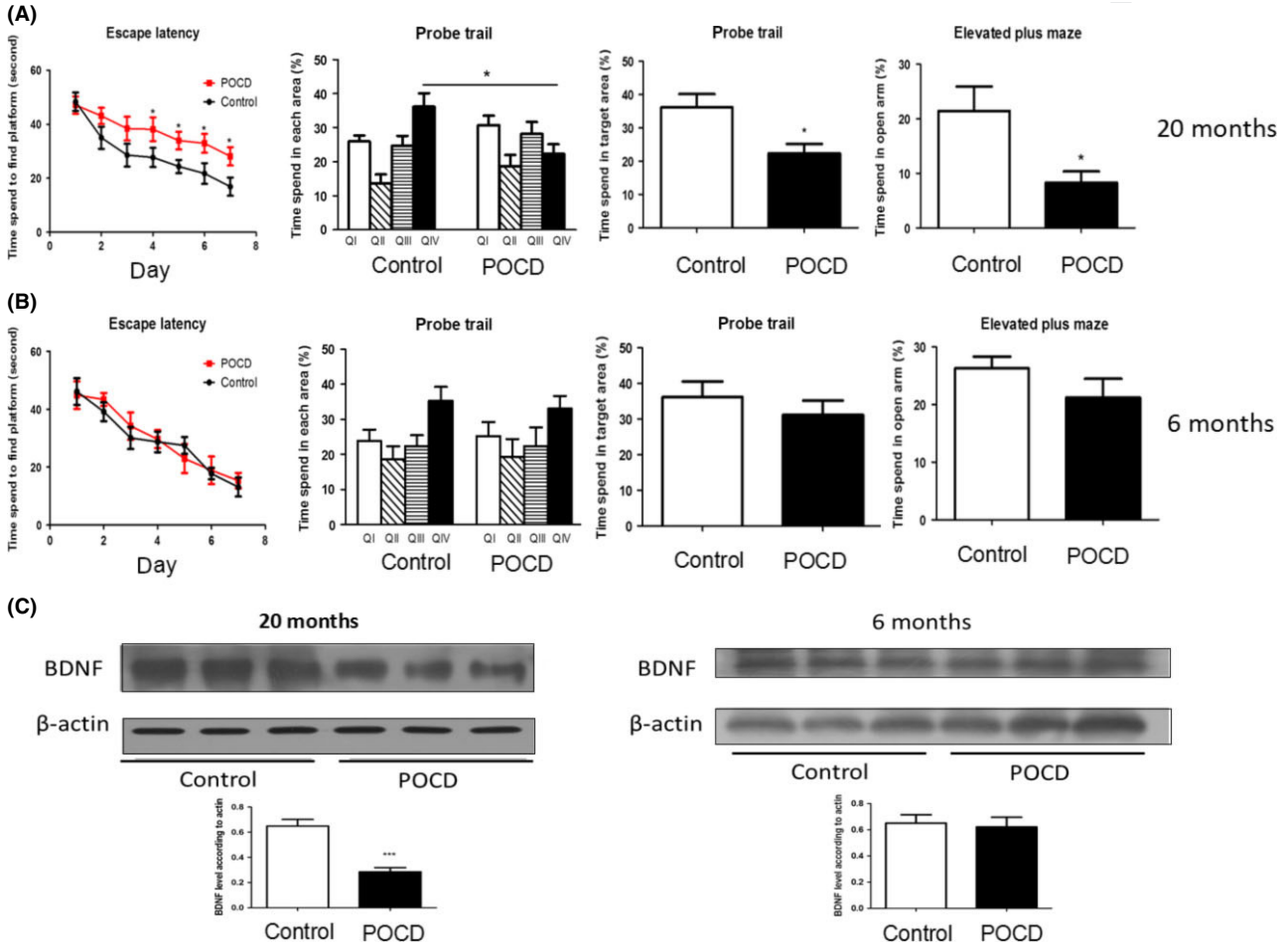

Figure 1 Surgical stress induces postoperative cognitive dysfunction in aged but not younger mice. (**A**), a seven-day escape latency trail for two groups of 20-month-old mice in Morris water maze test. POCD group spent longer latency to reach the hidden platform, compared with control mice (two-way ANOVA, treatment condition × day, $F(1,98) = 19.06$, $P < 0.001$ for treatment condition, POCD compared with control). The POCD group performed longer latency to reach the hidden platform on day 4, 5, 6, 7 (two-tailed unpaired *t*-test for comparisons in each day, $P < 0.05$). A probe trail with a removed platform was detected. POCD group displayed decreased time in target quadrant (Q IV) (two-tailed unpaired *t*-test, $P = 0.0129$). Elevated plus maze was detected in 20-month-old and 6-month-old mice. The time spent in open arm was shorter in POCD group at 20 months old (two-tailed unpaired *t*-test, $P = 0.0179$). (**B**), no significant difference on behavioral tests between POCD and control group at 6 months old. (Escape Latency:two-way ANOVA, treatment condition × day, $F(1,98) = 0.3392$, $P = 0.6261$ for treatment condition, POCD compared with control; two-tailed unpaired *t*-test for probe trail, $P = 0.2208$; elevated plus maze, two-tailed unpaired *t*-test, $P = 0.2133$). (**C**), the expression of BDNF level in prefrontal cortex was tested with Western blot in POCD and control mice. BDNF was downregulated in 20-month POCD group compared with control (two-tailed unpaired *t*-test, $P < 0.001$). No significant changes on BDNF level in 6-month groups (two-tailed unpaired *t*-test, $P = 0.7660$). (*$P < 0.05$, ***$P < 0.001$, POCD compared with control) (n = 8 mice/group). Error bar represent SEM.

participates in surgical stress-induced GR phosphorylation. The level of CDK5 was found elevated in 20-month-old POCD mice compared with control group ($P = 0.0178$) (Figure 3B). We also detected the level of CDK5 regulator p35 and p25 in POCD and control mice. Both p35 ($P = 0.0140$) and p25 ($P = 0.0139$) had an appreciable increase in POCD group compared with control group (Figure 3B). These data indicated that the increase of CDK5 and its regulator might be one of the reasons for the elevation of GR phosphorylation. The relationship between GR phosphorylation and cognitive dysfunction was also examined by correlation analysis conducted with Prism 5. CDK5 and pGRs220 both have negative correlation with behavioral index that represents cognitive function (CDK5 with MWM, $r^2 = 0.75$, $P < 0.001$; CDK5 with EPM, $r^2 = 0.78$, $P < 0.001$; pGRs220 with MWM, $r^2 = 0.63$, $P < 0.001$; pGRs220 with EPM, $r^2 = 0.73$, $P < 0.001$) (Figure 3C). The level of pGRs220 and CDK5 had no significant changes in 6-month-old mice ($P > 0.05$) (Figure 3D). These results indicated that GR phosphorylation might be a key difference between aged and young individuals in POCD.

Surgical Stress-Induced BDNF Reduction and Cognitive Dysfunction are Rescued by GR Antagonist

To demonstrate whether surgical stress-induced cognitive dysfunction was mediated by GR activation, we use a specific GR

LOW RESOLUTION FIG

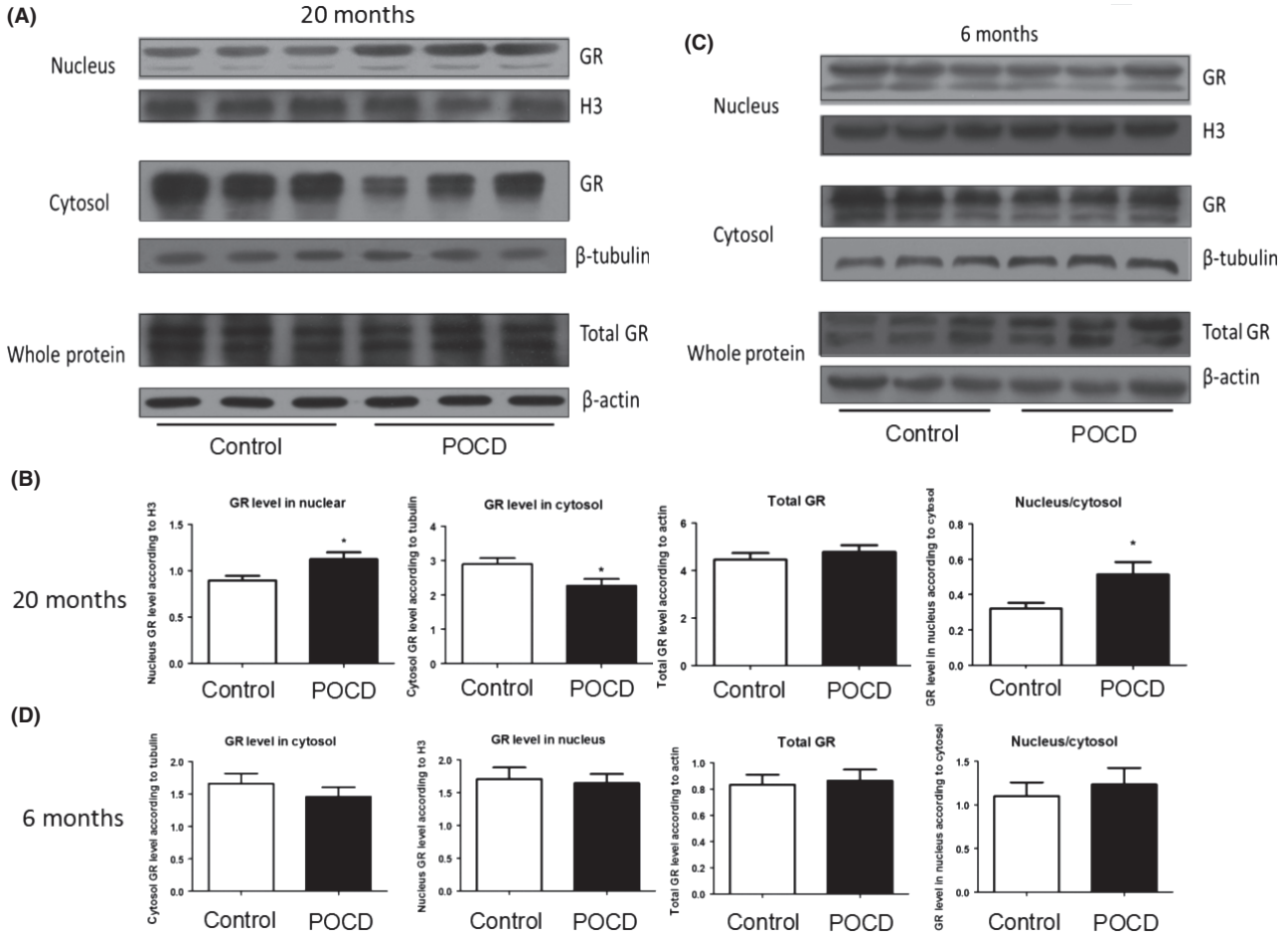

Figure 2 Surgical stress elevated glucocorticoid receptor function in nucleus in prefrontal cortex of POCD mice. (**A**) Glucocorticoid receptor level in nucleus and cytosol of prefrontal cortex were tested with Western blot in POCD and control mice. Glucocorticoid receptor in nucleus was upregulated in POCD group compared with control (two-tailed unpaired *t*-test, $P = 0.0253$). Oppositely, GR level in cytosol was decreased in POCD group (two-tailed unpaired *t*-test, $P = 0.0331$). The ratio of nucleus/cytosol GR significantly increased in POCD group compared with control (two-tailed unpaired *t*-test, $P = 0.0257$). Total GR in prefrontal cortex had no significant difference between two groups (two-tailed unpaired *t*-test, $P = 0.2371$). (**B**), quantification of Western blot in 20-month groups. (*$P < 0.05$, POCD compared with control) (n = 8 mice/group). Error bar represents SEM. (**C**), no significant changes on GR translocation in 6-month groups (two-tailed unpaired *t*-test, $P = 0.7874$ for GR in nucleus; $P = 0.3628$ for GR in cytosol; $P = 0.5937$ for ratio of nucleus/cytosol GR; $P = 0.7915$ for total GR). (**D**), quantification of Western blot in 6-month groups. (*$P < 0.05$, POCD compared with control) (n = 8 mice/group). Data are Mean ± SEM (Error bars).

antagonist RU486 on behavior tests in aged mouse. There was a significant effect of Ru486 on escape latency (two-way ANOVA, treatment condition × day, $F(1,98) = 20.15$, $P < 0.001$; POCD+Ru486 compared with POCD group by followed Tukey's *post hoc* test, $P < 0.001$). One-way ANOVA following with Tukey's *post hoc* test was used to detect other data for multiple groups. We found the POCD+RU486 group performed lower latency to reach the hidden platform ($P < 0.05$ on day 5, 6; $P < 0.01$ on day 7) (Figure 4A) and spent longer time in target area (Q IV) of removed platform ($P = 0.0189$) (Figure 4A) compared with POCD group in MWM tests. The swimming speed had no difference between all groups ($P = 0.8762$, POCD+RU486 compared with POCD) (Figure 4A). We also found POCD+RU486 group spent longer time in the open arm in elevated plus maze test ($P = 0.0388$) (Figure 4A) compared with POCD group. We also used RU486 to detect whether the BDNF reduction is induced by GR activation. We found BDNF level was rescued in POCD+RU486 group compared with POCD group ($P = 0.0354$) (Figure 4B). These data suggested that surgical stress-induced cognitive dysfunction and BDNF reduction were mediated by GR activation.

Surgical Stress-Induced GR Phosphorylation, BDNF Reduction, and Cognitive Dysfunction are Rescued by CDK5 Inhibitor

To demonstrate whether surgical stress-induced cognitive dysfunction was mediated by CDK5-induced GR phosphorylation, we use a specific CDK5 inhibitor roscovitine in behavior tests. There was a significant effect of roscovitine on escape latency (two-way ANOVA, treatment condition × day, $F(1,98) = 11.21$, $P < 0.001$; POCD+roscovitine compared with POCD group followed by

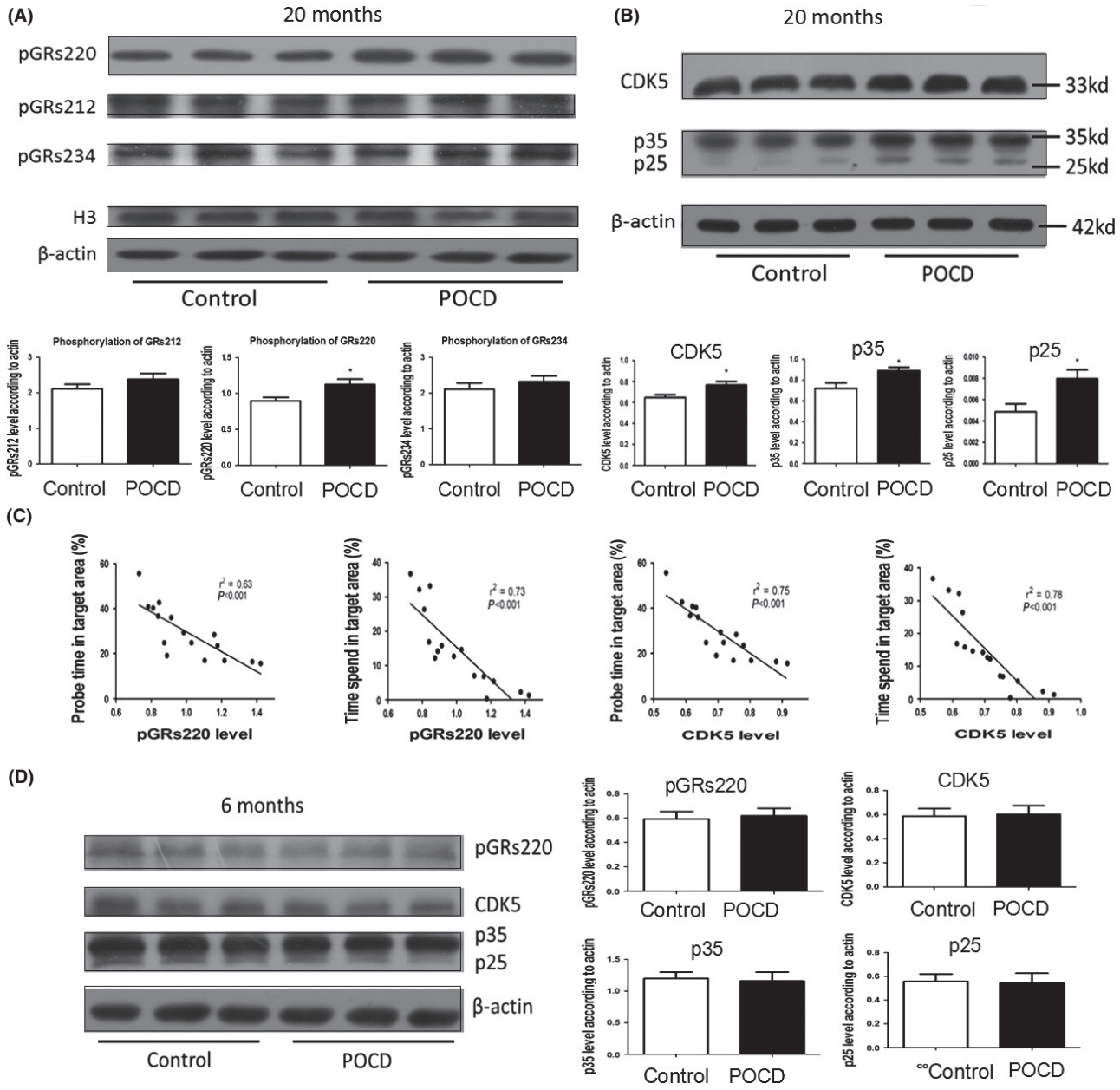

Figure 3 Surgical stress elevated GR phosphorylation at ser220 by CDK5 and its regulator in prefrontal cortex of POCD mice. (**A**), GR phosphorylation was tested with Western blot. The phosphorylation at ser220 site was significantly elevated in POCD group (two-tailed unpaired *t*-test, $P = 0.0136$ for pGRs220; $P = 0.2303$ for pGRs212; $P = 0.3860$ for pGRs234), compared with control group. (**B**) The level of CDK5 and its regulators p35, p25 in prefrontal cortex was tested with Western blot in POCD and control mice. The level of CDK5 was upregulated in POCD group compared with control (two-tailed unpaired *t*-test, $P = 0.0178$). CDK5 regulators p35 ($P = 0.0140$), p25 ($P = 0.0139$) also elevated in prefrontal cortex compared with control. (**C**), nonparametric correlation analysis of GR phosphorylation and behavioral index in 20-month groups (CDK5 with MWM, $r^2 = 0.75$, $P < 0.001$; CDK5 with EPM, $r^2 = 0.78$, $P < 0.001$; pGRs220 with MWM, $r^2 = 0.63$, $P < 0.001$; pGRs220 with EPM, $r^2 = 0.73$, $P < 0.001$) (**C**). (*$P < 0.05$, POCD compared with control) (n = 8 mice/group). Error bar represents SEM. (**D**), no significant changes on pGRs220 ($P = 0.7576$), CDK5 ($P = 0.8640$) and its regulators ($P = 0.8182$ for p35 and $P = 0.8844$ for p25) in 6-month groups. (*$P < 0.05$, POCD compared with control) (n = 8 mice/group). Data are Mean ± SEM (Error bars).

Tukey's *post hoc* test, $P < 0.001$). One-way ANOVA followed by Tukey's *post hoc* test was used to detect other data for multiple groups. We found that POCD+roscovitine group performed lower latency to reach the hidden platform ($P < 0.05$ on day 6, 7) (Figure 5A) and spent longer time in target area (Q IV) with removed platform ($P = 0.0446$) (Figure 5A) compared with POCD group in MWM tests. The swimming speed had no difference between all groups (POCD+roscovitine compared with POCD, $P = 0.8075$) (Figure 5A). We also found POCD+roscovitine group spent longer time in the open arm in elevated plus maze test ($P = 0.0199$) (Fig-

LOW RESOLUTION COLOR FIG

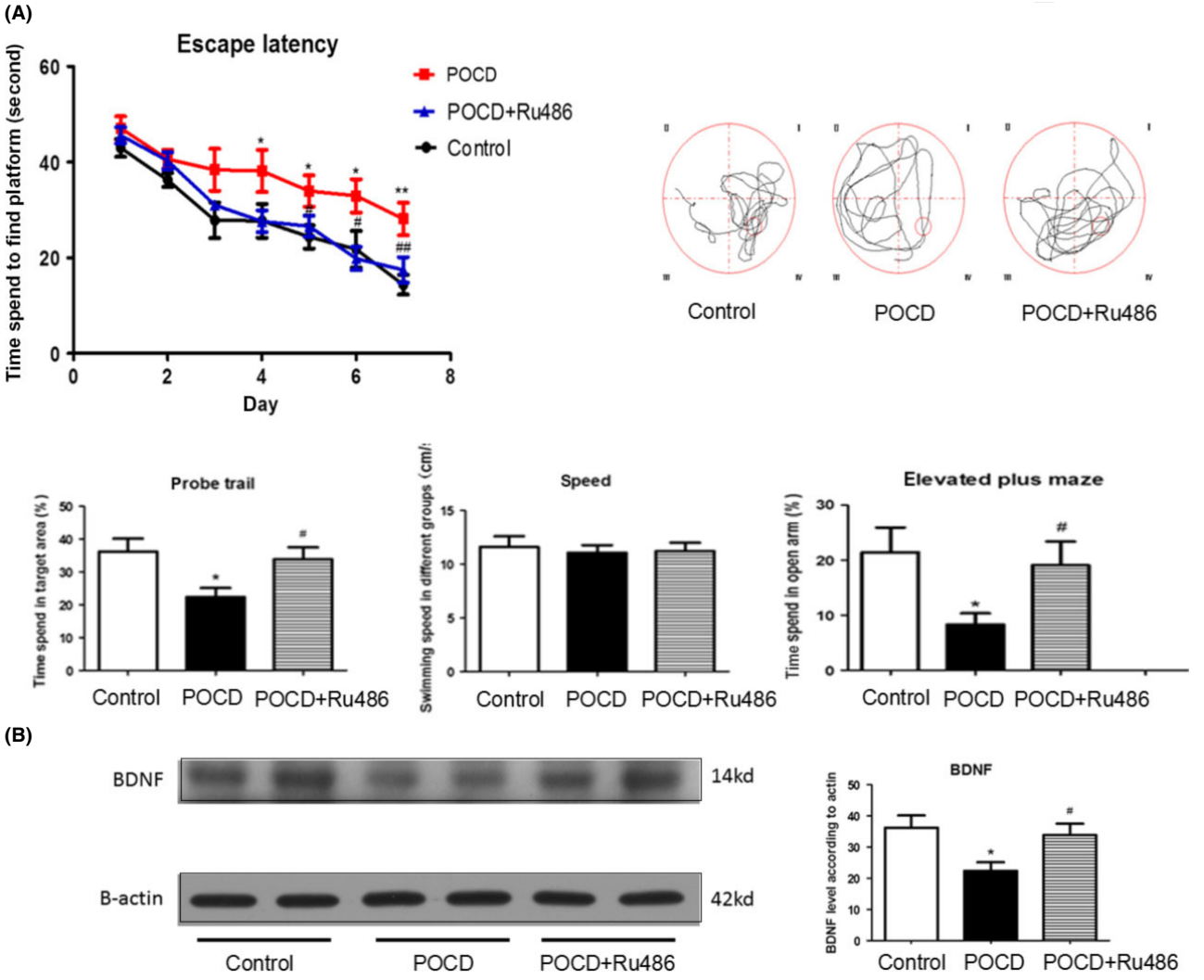

Figure 4 Surgical stress-induced BDNF reduction and cognitive dysfunction are rescued by GR antagonist. (**A**), Mifepristone, a specific GR antagonist was 26 treated just after the surgery. The performance on Morris water maze and elevated plus maze were rescued with treatment of RU486. POCD+RU486 group performed shorter latency to find the hidden platform (two-way ANOVA, treatment condition × day, $F(1,98) = 20.15$, $P < 0.001$; POCD+RU486 compared with POCD group followed by Tukey's *post hoc* test, $P < 0.001$; one-way ANOVA following with Tukey's *post hoc test* was used to compare latency on each day, $P < 0.05$ on day 5, 6 and $P < 0.01$ on day 7, POCD+Ru486 compared with POCD) and spent longer time in target area (Q IV) with a removed platform (one-way ANOVA following with Tukey's *post hoc test*, $P = 0.0189$), compared with POCD group. No significant difference on swimming speed among groups ($P = 0.8762$, POCD+Ru486 compared with POCD) and the swimming paths were also presented. In elevated plus maze test, time spent in open arm was also rescued with RU486 treatment (one-way ANOVA following with Tukey's *post hoc test*, $P = 0.0388$, POCD+Ru486 compared with POCD), compared with POCD group. (**B**), the expression of BDNF level in prefrontal cortex was also rescued in POCD+RU486 group compared with POCD group (one-way ANOVA following with Tukey's *post hoc test*, $P = 0.0354$). ($^{*}P < 0.05$, POCD compared with control; $^{\#}P < 0.05$, POCD+RU486 17 compared with POCD) (n = 8 mice/group). Data are Mean ± SEM (Error bars).

ure 5A) compared with POCD group. We also used roscovitine to detect whether the BDNF reduction is mediated by CDK5 induced GR phosphorylation. The elevation of GR phosphorylation at ser220 was rescued by roscovitine ($P = 0.0277$) (Figure 5B). The level of BDNF was also rescued in POCD+roscovitine group compared with POCD group ($P = 0.0162$) (Figure 5B). These results indicated that the BDNF reduction in POCD was mediated by CDK5-induced GR phosphorylation. These data also suggested that surgical stress-induced cognitive dysfunction and BDNF reduction were mediated by CDK5-induced GR phosphorylation.

Surgical Stress Induces Sustaining GR Phosphorylation in Aged but not in Young Mouse

We also collected samples on day 1 and 13 postoperation (postop) to detect GR phosphorylation in different time points. The level of

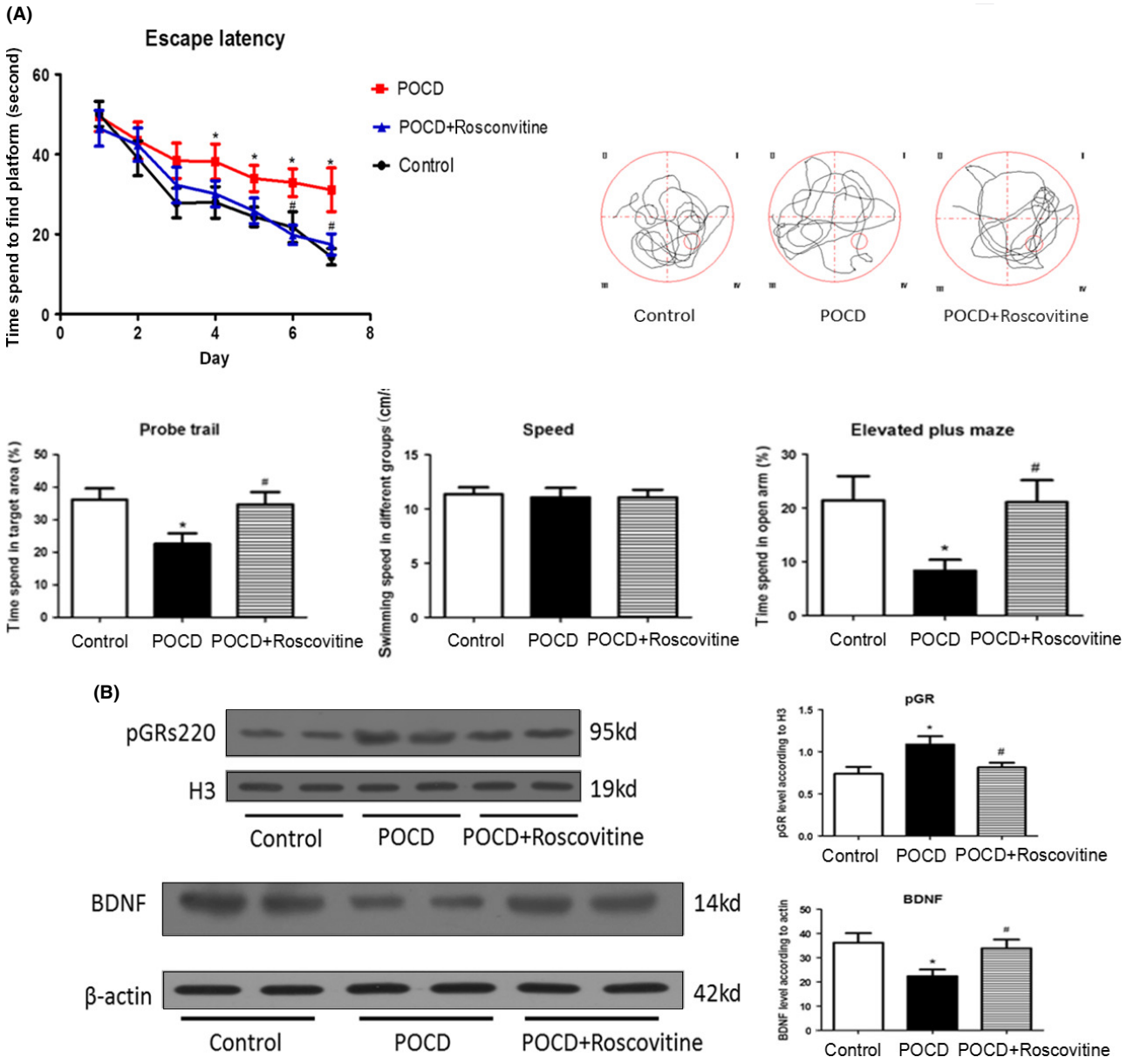

Figure 5 Surgical stress-induced GR phosphorylation, BDNF reduction and cognitive dysfunction are rescued by CDK5 inhibitor. (**A**) Roscovitine, a specific CDK5 inhibitor was treated after the surgery. The performance on Morris water maze and elevated plus maze was rescued with treatment of roscovitine. POCD+roscovitine group performed shorter latency to find the hidden platform (two-way ANOVA, treatment condition × day, $F(1,98) = 11.21$, $P < 0.001$; POCD+roscovitine compared with POCD group followed by Tukey's *post hoc* test, $P < 0.001$; one-way ANOVA following with Tukey's *post hoc* test was used to compare latency on each day, $P < 0.05$ on day 6 and 7, POCD+roscovitine compared with POCD) and spent longer time in target area with a removed platform (one-way ANOVA following with Tukey's *post hoc test*, $P = 0.0446$), compared with POCD group. No significant difference on swimming speed among groups ($P = 0.8075$, POCD+roscovitine compared with POCD) and swimming paths were also presented. In elevated plus maze test, time spent in open arm was also rescued with roscovitine treatment (one-way ANOVA following with Tukey's *post hoc test*, $P = 0.0199$, POCD+roscovitine compared with POCD). (**B**), the level of GR phosphorylation at ser220 site in nucleus was rescued in POCD+roscovitine group compared with POCD ($P = 0.0277$). The expression of BDNF level in prefrontal cortex was also rescued in POCD+roscovitine group compared with POCD ($P = 0.0162$). ($^{*}P < 0.05$, POCD compared with control; $^{\#}P < 0.05$, POCD+roscovitine compared with POCD) (n = 8 mice/group). Data are Mean ± SEM (Error bars).

pGRs220 in control groups was set as 100% to compare the elevation of pGRs220 in POCD groups. On day 1 postop, GR phosphorylation at ser220 was found elevated in both aged ($P = 0.009$) and younger POCD mice ($P = 0.0228$) compared with each control groups (Figure 6A & B). On day 13 postop, pGRs220 level in younger group was recovered ($P = 0.6822$) (Figure 6B). However, GR phosphorylation at ser220 was still at a high level in aged POCD mouse on day 13 postoperation ($P = 0.0187$) compared

LOW RESOLUTION FIG

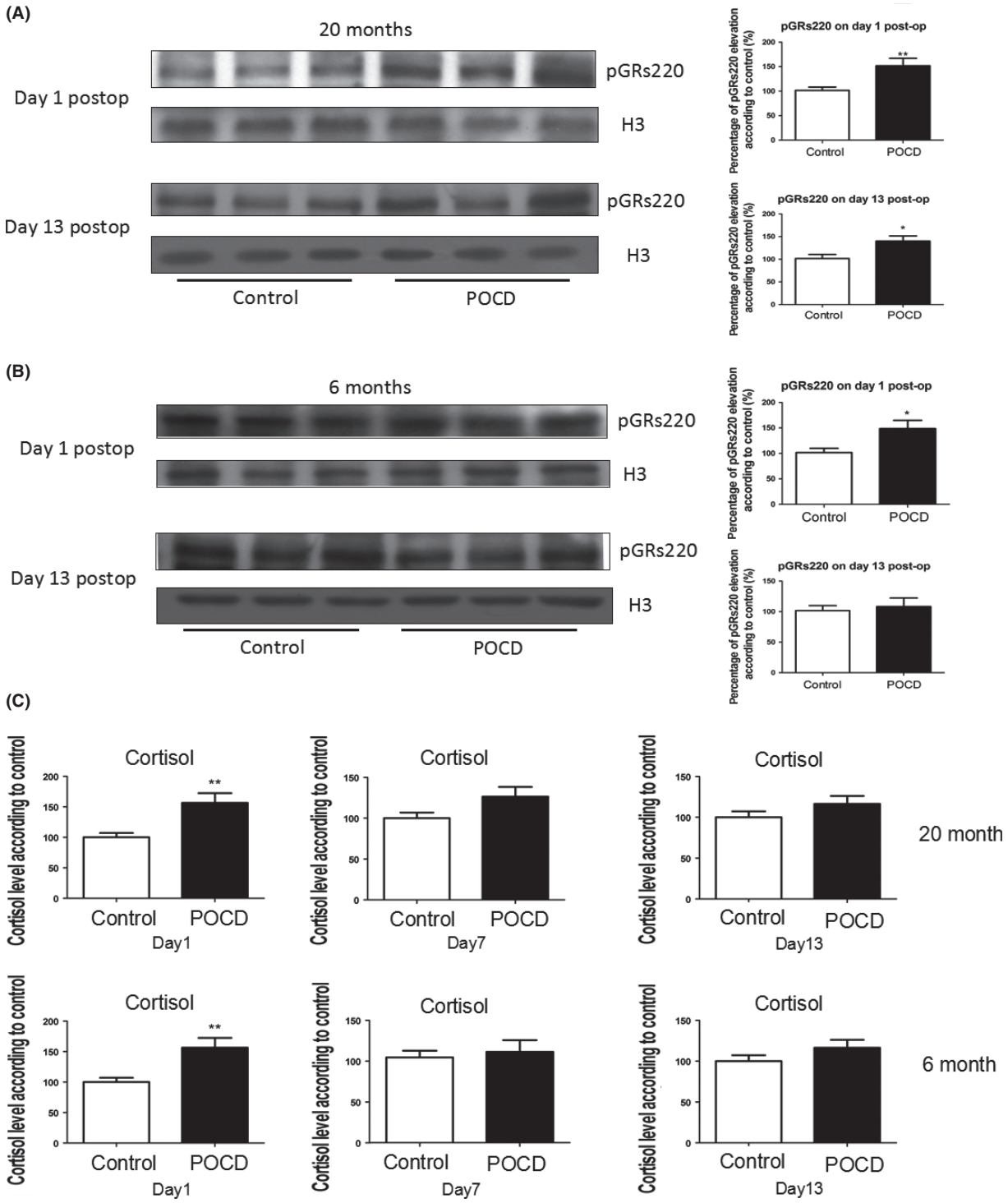

Figure 6 Surgical stress induces sustaining GR phosphorylation in aged but not in young mouse. (**A** & **B**), we used Western blot to detect the pGRs220 level in prefrontal cortex of 20-month-old and 6-month-old mouse. The samples were collected on day 1 and 13 after surgery. The level of pGRs220 was compared to the control group on each time point. The percentage of pGRs220 elevation was presented. On day 1 postop, pGRs220 was elevated in POCD groups compared with each control, in both 20 (two-tail unpaired *t*-test, $P = 0.0090$, elevated by 51.9%) and 6 (two-tail unpaired *t*-test, $P = 0.0228$, elevated by 48.2%) months of mouse. On day 13 postop, pGRs220 was still at a high level in 20-month-old group, compared with control ($P = 0.0187$). In 6-month-old groups, no significant difference on pGRs220 level between POCD and control mouse ($P = 0.6822$). (**C**), plasma cortisol level compared to control groups in both aged and young mouse.(*$P < 0.05$, POCD group compared with control group; C, control; P, POCD; postop, postoperation) (n = 8 mice/group). Data are Mean ± SEM (Error bars).

with control group (Figure 6A). Plasma cortisol level was also detected in both ages on day 1, 7, and 13 postop (Figure 6C). The cortisol level elevated in early stage and recovered gradually ($P < 0.01$ on day 1 postop). These data indicated that cortisol might be involved in the elevation of GR phosphorylation in early stage. However, the sustaining GR phosphorylation is cortisol-independent.

Discussion

We first found GR translocation into nucleus and elevated pGRs220 in prefrontal cortex of aged but not younger POCD groups. Then, we demonstrated the reduction of BDNF expression and cognitive dysfunction after surgical stress. The reduction of BDNF and cognitive dysfunction was rescued by GR antagonist and CDK5 inhibitor. These data indicated that stress-induced GR phosphorylation might be a significant neuropathology and treatment target of POCD.

GR Activation is Mediated by both Ligand and Kinase

A recent study has reported that the level of cortisol, a primary glucocorticoid, was increased in plasma of POCD patients [18]. As binding to cortisol, GR will be phosphorylated by CDK5 and translocated into nucleus from cytosol to regulate transcription [31]. Therefore, ligand (cortisol) and kinase (CDK5 as a representative) could be a double control to the phosphorylation of GR. In our study, we use two kinds of inhibitor to rescue the phosphorylation of GR, RU486, and roscovitine. RU486 is a kind of GR antagonist which could competitively bind to GR with cortisol [32]. Roscovitine is a kind of CDK5 inhibitor. Consistent with our hypothesis, both of the two inhibitors rescued BDNF reduction and postoperative cognitive dysfunction. Moreover, Ru486 and roscovitine have multiple substrates including GR and CDK5. These results presented a phenomenon that GR signaling was involved in POCD. We also found that plasma cortisol elevated in early stage after surgery and recovered gradually (Figure 6C). However, sustaining GR phosphorylation lasted 1–2 weeks in aged groups (Figure 6A & B). These results indicated that surgical stress-induced cortisol elevation might be involved in GR activation in early stage. Yet, it may have little effects on sustaining of GR phosphorylation in late period after surgery which probably is controlled by other mechanisms.

GR Activation and Inflammation

Lots of researches report that GR was closely related to inflammation [32–36], and surgery-induced inflammation probably leads to dementia and POCD [37,38]. As for surgical stress, activation of inflammation-associated NF-kB pathway and elevation of the downstream pro-inflammatory cytokines are closely linked to the incidence of cognitive impairment [37,39]. We also detect IL-1β, IL-6 and TNF on day 7 after surgical stress. These inflammatory factors were elevated in aged POCD group (Fig. S5), and the results were similar to the work mentioned above [38,39]. The elevation of inflammatory factors was probably a direct effect of surgical stress or an indirect effect by surgical stress–GR–inflammation circle. There is no doubt that GR and inflammation have a closely interaction, and the relationship between GR and inflammation in POCD should be studied further.

Transcription Regulation by GR Phosphorylation Participates in Cognitive Dysfunction and Stress

Glucocorticoid and its receptor are closely related to stress and cognition [10,40,41]. Activation of GR by phosphorylation following behavioral or cellular stress [17] controls transcription of genes participates in stress and cognitive dysfunction. In this work, we detected GR phosphorylation in three common sites as representative. The ser220 site is one of the most important sites for GR activation and its downstream functions. However, GR has lots of modifiable sites, and GR activation is a complex progress [42]. Much more work need to be carried out to discover its beyond mechanism. Contribute to the survival of neuron and synapse, BDNF is often correlated with memory, stress, emotion, and dementia [28,29]. A large number of researches demonstrate that GR has close relationship to BDNF [39,42,43]. Dexamethasone (DEX), a potent GR agonist could inhibit the expression of BDNF *in vivo* and *in vitro* [44,45]. A recent study found BDNF decreased in POCD animal models [30]. This result suggested that BDNF was involved in POCD. Another study found that BDNF level was not changed in peripheral samples of patients with POCD [46]. However, in parenchyma, the level of BDNF is found decreased after surgery [30]. Much more work is to be carried out to discover the exactly mechanism. In our 18
study, the level of BDNF was reduced in POCD group and was rescued by GR phosphorylation inhibition. These data probably suggested that surgical stress-induced GR activation could impaired the postoperative cognitive function via BDNF. Recent studies reported that formaldehyde was related to POCD and phosphorylation [47,48]. Formaldehyde elevation is 19
one of the changes after surgical stress. Another study reported that formaldehyde is related to CDK5, which participated in GR phosphorylation [49]. This does not contradict with our finding. GR activation probably is induced by several changes after surgical stress such as formaldehyde, cortisol, and inflammatory factors. The relationship between formaldehyde stress and GR activation is an interesting topic that needs to be studied in future.

Sustaining GR Phosphorylation in Elder but not in Younger Patients

The morbidity of POCD is closely related to the age of patients who had clinical surgery [3,4]. So, what is the difference between elder and younger patients in pathological process of POCD? In this work, we found elevated GR phosphorylation in both ages on early stage after surgery. However, sustaining GR phosphorylation in late period after surgery only existed in aged groups and was cortisol-independent (Figure 6). Large numbers of clinical researches

demonstrated that POCD could last several weeks or even months in elder patients [50]. According to these results, we hypothesized that the ability of self-regulation on HPA axis might be the difference between elder and younger individuals.

Surgical Stress-Induced POCD in a Laparotomy Animal Model

Different models have been used in POCD studies, inhalation anesthesia and various kinds of surgical style included [23,51–54]. In this research, we chose laparotomy to exclude the effects of surgical style and viscera injury and the general incidence of POCD was reported to have little difference between the types of surgeries [55]. A few studies also attach great importance of the effects of surgical incision itself [23,56]. Moreover, the kinase of GR phosphorylation, CDK5, was reported increased in the model of isoflurane inhalation [19]. Therefore, we used a general anesthesia with chloral hydrate intraperitoneal injection instead for excluding the effects of isoflurane.

Conclusion

Our data indicated that surgical stress-induced BDNF reduction and cognitive dysfunction were mediated by GR phosphorylation in aged mice. These results suggested that surgical stress-induced GR activation and sustaining phosphorylation might be a potential mechanism and treatment target of POCD.

Acknowledgments

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Conflict of Interest

The authors declare no competing financial interests.

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Supporting Information

The following supplementary material is available for this article:

Figure S1. XXXXX.
Figure S2. XXXXX.
Figure S3. XXXXX.
Figure S4. XXXXX.
Figure S5. XXXXX.
Figure S6. XXXXX. 22

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