

Roles of c-Fos, EGR-1, PKA, and PKC in cognitive dysfunction in rats after propofol anesthesia

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This study aimed to investigate possible changes in the spatial memory of rats and the expression or activity of EGR-1, c-Fos, PKA, and PKC after propofol anesthesia. Thirty-six Sprague-Dawley rats aged 20 months and 36 Sprague-Dawley rats aged three months were each randomly divided into three groups: the control group, the Morris Water Maze (MWM) group, and the propofol group. In the propofol groups of both young and aged rats, the rats were anesthetized by propofol for two or four hours and then performed the MWM test two days or two weeks after anesthesia to assess cognitive function. EGR-1, c-Fos, PKA, and PKC expressions in the rat hippocampus were determined via immunohistochemistry. For the older rats, the escape latency in the P_{4h/2d} group was significantly prolonged ($P < 0.05$), and the learning curve was right-shifted in the P_{4h/2w} group ($P < 0.05$). The expression levels of EGR-1, c-Fos, PKA, and PKC in the MWM groups were significantly higher than those in the control groups ($P < 0.05$). In the P_{4h/2d} group of aged rats, the expression levels of both PKA and PKC were decreased compared with those of the MWM groups. The decreased expression of both protein kinases may be responsible for the observed impairment after propofol anesthesia.

Keywords: Propofol. Hippocampus. c-Fos. EGR-1. PKA. PKC.

INTRODUCTION

Cognitive impairment following operation and anesthesia is a complex process that can last for weeks and is defined as post-operative cognitive dysfunction (POCD). Aging is now generally accepted to be responsible for POCD. Anesthesia is a possible cause as well (Tachibana *et al*, 2015). Studies about POCD after propofol administration produced inconsistent results, and the underlying mechanism remains to be revealed.

Neuronal immediate-early gene (IEG) expression plays an important role in the neuroplastic mechanisms critical to memory (Carter, Mifsud, Reul, 2015). It is closely related to the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinases (MAPK) pathway (Besnard *et al*, 2011). In hippocampus slices,

propofol induces *c-fos* transcription but decreases *Egr-1* transcription (Kidambi, 2010). In our previous study using a proteomic technique, differential expression of MAPK1 in the rat hippocampus was detected until seven days after propofol anesthesia (Zhang *et al*, 2009). However, the implications of these effects on POCD remain to be investigated.

IEG expression levels are regulated by protein kinases. For example, the expression of EGR-1 and c-Fos requires a combination of protein kinases, including cyclic adenosine monophosphate (cAMP) and dependent protein kinase (PKA) (Simpson, Morris, 1995). Calcium/phospholipids-dependent protein kinase C (PKC) increases c-Fos expression, which is a marker of neural activity (Narita *et al*, 2004). Both PKA and PKC are critical to memory and cognitive dysfunctions (Takigami *et al*, 2014; Zhong *et al*, 2018). However, little is known about the effects of propofol on protein kinases, especially their roles in POCD.

Considering these results, we postulated that both protein kinases and the products of IEGs might be associated with cognitive dysfunction after anesthesia.

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Therefore, we aimed to explore possible changes in spatial memory in rats post propofol anesthesia using the Morris Water Maze (MWM) test and to elucidate the underlying molecular mechanisms by observing the expression of EGR-1, c-Fos, PKA, and PKC in the CA1 subfield of the hippocampus.

METHODS

Rat grouping and anesthesia

The surgical inventions and animal care procedures were all approved by the Laboratory Animal Welfare and Ethics Committee of our institute, and the procedures were in accordance with the guidelines laid down by the National Institute of Health in the USA. Based on the duration of anesthesia, the age of the rats, and the time-points of behavioral testing post-anesthesia, 36 Sprague-Dawley (SD) rats aged 20 months (375 ± 25 g) and 36 SD rats aged three months (200 ± 20 g) were each randomly divided into three groups: the control group, the MWM group, and the propofol group. In the propofol groups of both young and aged rats, the rats were anesthetized by propofol for two or four hours. they performed the MWM test two days or two weeks after anesthesia to assess spatial memory (six rats/subgroup: $P_{2h/2d}$, $P_{4h/2d}$, $P_{2h/2W}$, and $P_{4h/2W}$).

Propofol (2, 6-diisopropylphenol) was administered intraperitoneally with an initial bolus injection (100 mg kg^{-1}) followed by a supplement dose when necessary, usually half of the initial bolus approximately one hour after the first injection.

Morris water maze training and tests

On the day before the MWM training, the rats were placed in the pool without a platform to swim freely for 120 s. MWM tests were performed for five days, with four training trials in the morning and four in the afternoon each day. The platform was placed in the south-west quadrant of the pool. The rats began their trials from four different quadrants and cycled throughout the four trials each half-day, with each trial lasting a maximum of 120 s. They were allowed to stay on the platform for 10–30 s before the next trial. If the rats failed to find the

platform within 120 s, they would be manually guided to the platform and allowed to rest on the platform for 30 s before the next trial.

Escape latency and swim paths were tracked using an image auto-monitor system. A daily mean for the performance of each rat was obtained through averaging the eight daily trials. The testing environment and spatial cues were left undisturbed throughout the experiment.

Immunohistochemical staining

The rats were transcardially perfused with 300–400 mL 0.1 mol/L phosphate buffer solution (PBS) immediately after the last MWM trial, followed by another perfusion with 300–400 mL 4 % paraformaldehyde in 0.1 mol/L PBS (pH 7.4). The brains were isolated and cryoprotected in a 30 % sucrose solution containing 4 % paraformaldehyde at 4 °C overnight. Afterward, all brains were sectioned on a Cryotome E freezing slice machine. The hippocampus CA1 subfield was localized within 3.0–3.5 mm posterior to the anterior fontanelle. Sections (35- μm thick) of the CA1 subfield were obtained and every fifth section was saved for immunohistochemical analysis.

The slices were rinsed three times in 0.01 mol/L phosphate buffer saline and then treated for 30 minutes in PBS containing 0.3 % Triton. For hematoxylin and eosin and immunohistochemical staining, the slices were incubated with a solution containing rabbit antibodies for EGR-1, c-Fos, PKA, and PKC (Santa Cruz Biotechnology Biotechnology, CA, USA 1:1000) at 37 °C for four hours and then stored at 4 °C overnight. Subsequently, the sections were incubated in an antiserum of anti-rabbit IgG (1:500) at 37 °C for four hours, followed by incubation at room temperature with an avidin-biotin-HRP complex for one hour. Staining was visualized using freshly-prepared DAB.

Subsequently, the sections were rinsed three times with PBS, mounted onto slides, dried, and dehydrated in solutions with ascending alcohol concentrations. Five sections (four fields/section) of the hippocampal CA1 subfield for each rat were observed under an optical microscope. All slices were analyzed at 400x magnification under identical luminous intensities. The

average numbers of EGR-1, c-Fos, PKA, or PKC cells were calculated within each field of vision.

Statistical analyses

Data were processed by the SPSS 12.0 software package (SPSS, USA). Between- and within-group comparisons were made using one-way ANOVAs. Results were considered significant if $P < 0.05$.

RESULTS

Morris water maze tests

There were no significant differences in the swimming speed between groups of either young or

aged rats. The impairments of spatial memory were assessed as increased escape latencies during training and/or poor performance in the probe trials. Specifically, propofol anesthesia did not significantly influence MWM performance of young rats, with the escape latency being stable after day 3 (Figure 1).

For aged rats, the escape latency of the $P_{4h/2d}$ group was significantly increased compared with the MWM group ($P < 0.05$). In the $P_{4h/2w}$ group, the escape latency was not increased ($P > 0.05$). However, the learning curve was right-shifted ($P < 0.05$), which means that the escape latency was stable until day 4. The escape latency in the $P_{2h/2d}$ and $P_{2h/2w}$ groups was not affected by propofol ($P > 0.05$, Figure 2).

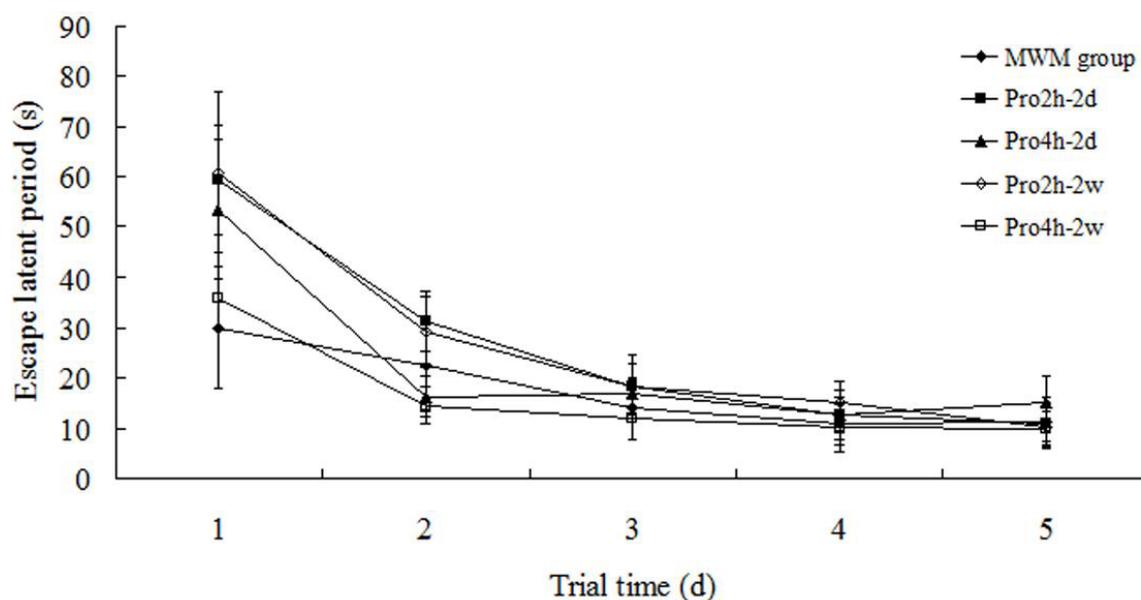


FIGURE 1 - Morris water maze training in young rats treated with propofol. Comparing with MWM group, the performance of young rats treated anesthetized by propofol of either 2h or 4h was not significantly impaired. The escape latent period was stable after day 3 ($P < 0.05$).

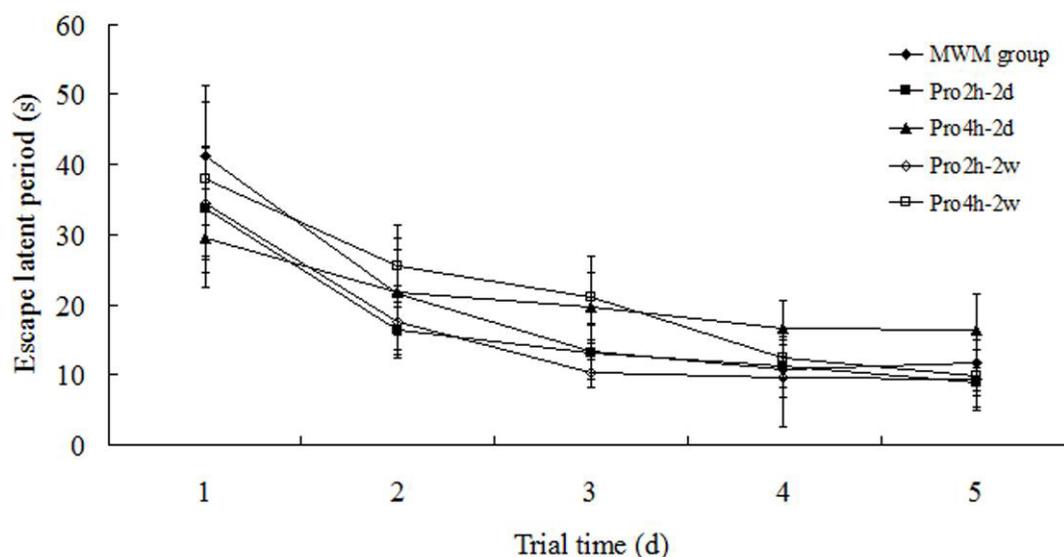


FIGURE 2 - Morris water maze training in aged rats treated with propofol. Comparing with MWM group, the escape latent period of P4h/2d group was significantly increased ($P < 0.05$). The escape latent period was stable until day 4 in P4h/2w group, which means the learning curve was right-shifted ($P < 0.05$). Escape latency in group P2h/2d and P2h/2w was not affected by propofol ($P > 0.05$).

Expression of EGR-1, c-Fos, PKA, and PKC

Few EGR-1 or c-Fos and no PKA- or PKC-positive cells were detected in the hippocampal CA1 region of rats in the control groups of both young and aged rats. However, the expression of all proteins was significantly increased after MWM training ($P < 0.05$).

The expression of EGR-1 and c-Fos was similar in the MWM groups of both young and aged rats ($P > 0.05$, Figure 3 and 4).

The expression of PKA and PKC in the propofol group of young rats was similar to those in the MWM group ($P > 0.05$), whereas the expression was decreased in the P_{4h/2d} group of aged rats compared with that of the MWM group (Figure 5 and 6).

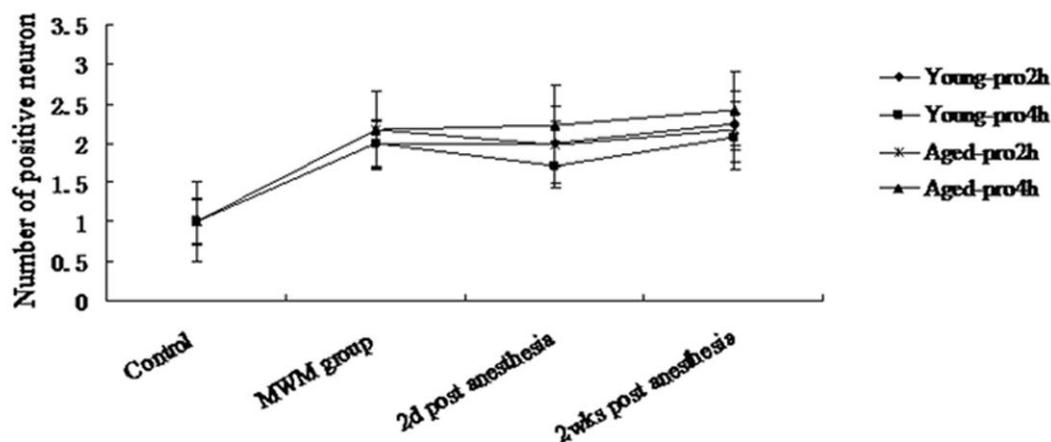


FIGURE 3 - Effect of propofol anesthesia on the expression of EGR1. Few EGR1 were seen in the hippocampal CA1 area of both young and aged rats. In MWM groups of all rats, number of EGR1 positive neurons in this area was significantly increased ($P < 0.05$), which was not affected by propofol exposure ($P > 0.05$).

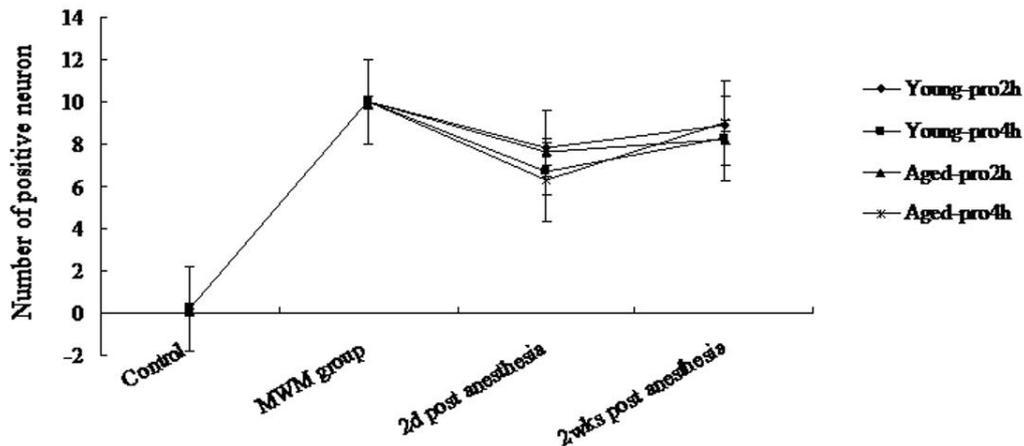


FIGURE 4 - Effect of propofol anesthesia on the expression of c-Fos. Few c-Fos were seen in the hippocampal CA1 area of both young and aged rats. In MWM groups of all rats, number of c-Fos positive neurons in this area was significantly increased ($P < 0.05$), which was not affected by propofol exposure ($P > 0.05$).

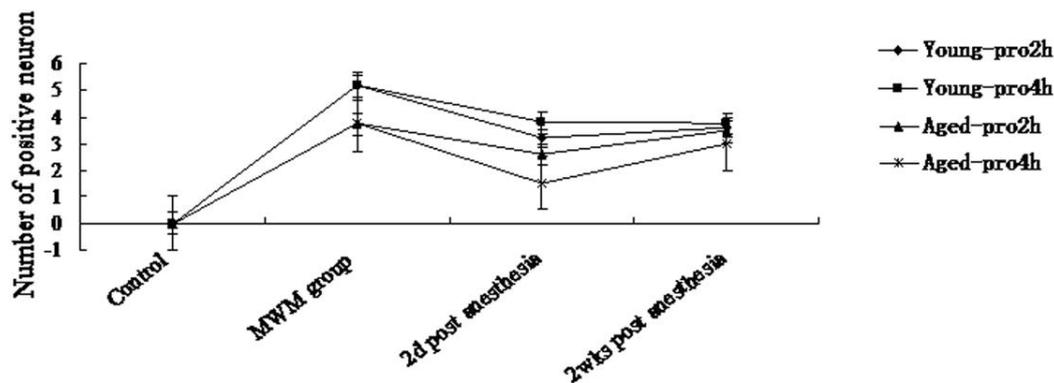


FIGURE 5 - Effect of propofol anesthesia on the expression of PKA. No PKA were seen in the hippocampal CA1 area of both young and aged rats. In MWM groups of all rats, number of PKA positive neurons in this area was significantly increased ($P < 0.05$), which was not affected by propofol exposure in young rats and aged rats in group P4h/2w ($P > 0.05$). The expression of PKA was depressed in aged rats of group P4h/2d comparing with those in MWM group ($P < 0.05$).

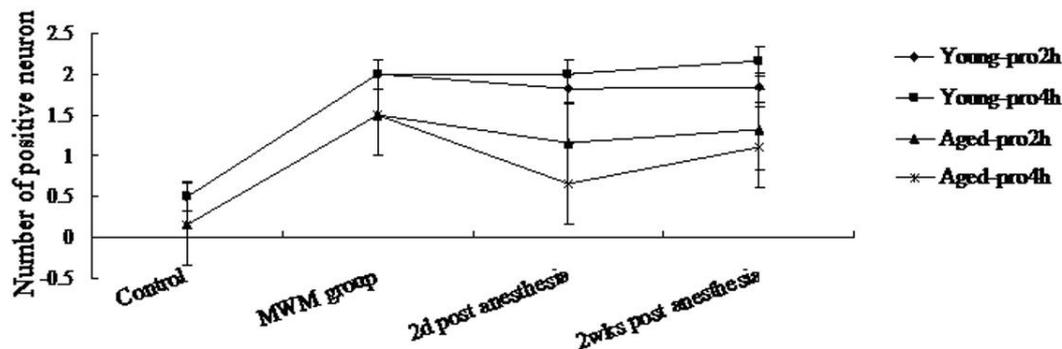


FIGURE 6 - Effect of propofol anesthesia on the expression of PKC. No PKC were seen in the hippocampal CA1 area of both young and aged rats. In MWM groups of all rats, number of PKC positive neurons in this area was significantly increased ($P < 0.05$), which was not affected by propofol exposure in young rats and aged rats in group P4h/2w ($P > 0.05$). The expression of PKC was depressed in aged rats of group P4h/2d comparing with those in MWM group ($P < 0.05$).

DISCUSSION

Cognitive impairments usually occur during early stage post propofol anesthesia (Schoen *et al*, 2011) but are also reported to have long-lasting effects on cognition (Han *et al*, 2015). This was supported by the present study because we found that the learning curve was right-shifted until two weeks after four-hour anesthesia in aged rats, implying prolonged impairment in the spatial memory after propofol anesthesia.

Since the impaired memory in our study was observed in the propofol group of aged rats, senility as the most important cause for cognitive dysfunction after anesthesia was verified. Moreover, although propofol is eliminated quickly and its effect disappears shortly even after long anesthesia, impairments in spatial memory were exclusively observed in aged rats anesthetized for four hours. Therefore, old age seems to be more sensitive to long periods of propofol anesthesia.

In the hippocampal CA1 region of both young and aged rats in this study, EGR-1 and c-Fos were significantly upregulated after MWM training, which is consistent with previous reports showing that IEG expression is regulated by synaptic activity which plays an important role in neuroplastic mechanisms critical to memory consolidation (Carter, Mifsud, Reul, 2015). However, the increased expression of both EGR-1 and c-Fos was not affected by propofol anesthesia, implying that they were not involved in the decreased spatial memory observed in the present study.

Both PKA and PKC play important roles in long-term potentiation (LTP) through protein or peptide phosphorylation (Rodríguez-Durán, Escobar, 2014; Liu *et al*, 2017). Specifically, PKC is involved in the early induction phase through phosphorylating glutamate receptor 1 subunits (GluR1) of α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors, whereas PKA is crucial for the late protein synthesis-dependent phase through phosphorylating MAPK (Boehm *et al*, 2006; Waltereit, Weller, 2003). In this study, PKA and PKC were upregulated in the hippocampal CA1 region of rats in the MWM groups, implying an enhancement in the signal transduction for LTP and the significant roles of both kinases in spatial memory.

Downregulation of PKC activity was discussed in another study involving memory impairment (Arya *et al*, 2016). Moreover, both PKC and PKA are subject to senile alteration. For example, a subtype of PKC in CA1 is correlated positively with spatial memory impairment among aged rats (Colombo, Gallagher, 2002) and the PKA-dependent pathway for LTP is decreased in hippocampal neurons from an Alzheimer's disease mouse model (Vitolo *et al*, 2002). In this study, the expression and activities of both PKC and PKA were decreased in aged rats of the P_{4h/2d} group, where the impairment of spatial memory was also observed, indicating that these two kinases might be responsible for POCD induced by prolonged anesthesia of propofol.

We recently reported that synaptic plasticity was impaired after propofol anesthesia (Li *et al*, 2015). Studies about cognitive kinases suggest that depressed expression and function of PKA and PKC are associated with synaptic dysfunction (Russo *et al*, 2018; Sacai *et al*, 2014). There are various molecules functioning as downstream targets of kinases during synaptic plasticity of memory, for example, c-Fos and EGR-1. Both belong to the regulatory transcription factors sub-class of IEG, which regulates not only synaptic function but also cell function globally. Considering that they were not affected in the present study, we postulate that propofol-related POCD is the result of synaptic dysfunction induced by specific pathways including PKA and PKC rather than global dysfunction of neurons. In fact, we have found evidence about the reorganization of actin as the result of upregulation of both Cofilin 1 and its phosphorylation after propofol anesthesia. Cofilin 1 is downstream of both PKA and PKC, which have complex effects on actin polymerization and synaptic plasticity. Therefore, the role of the PKA/PKC-cofilin pathway in POCD requires further investigation.

In this study, we aimed to detect cognitive dysfunction after propofol anesthesia and investigate the underlying mechanisms. Although several elements have been revealed, others remain to be explored. First, subtypes of PKC and PKA have different functions. Modifying these subtypes will help to elucidate the mechanisms discussed. Secondly, responses of the other members in this signal pathway should be also

investigated to support the results of this study. Finally, the results of this study do not explain the mechanisms of decreased memory until two weeks after anesthesia in aged rats.

CONCLUSION

The impairment of spatial memory induced by propofol is mostly seen in aged rats after long-term anesthesia and the impairments last at least two weeks post anesthesia. The downregulation of both PKC and PKA might be responsible for the observed impairment post propofol anesthesia, whereas EGR-1 and c-Fos might not be involved.

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