Neuronal mechanisms of adenosine A_{2A} receptors in the loss of consciousness induced by propofol general anesthesia with functional magnetic resonance imaging (fMRI)

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Abstract

Propofol is the most common intravenous anesthetic agent for induction of anesthesia and maintenance and has been clinically used for more than 30 years. However, the mechanism by which propofol induces loss of consciousness (LOC) remains largely unknown. The adenosine A_{2A} receptor ($A_{2A}R$) has been extensively shown to have an effect on physiological sleep. It is, therefore, important to investigate the role of $A_{2A}R$ in the induction of LOC using propofol. In the present study, the administration of the highly selective $A_{2A}R$ agonist (CGS21680) and antagonist (SCH58261) were utilized to investigate the function of $A_{2A}R$ under general anesthesia induced by propofol with the help of animal behavior studies, resting state magnetic resonance imaging (rsfMRI) and *c*-Fos immunofluorescence staining approaches. Results show that CGS21680 significantly prolonged the duration of LOC induced by propofol, increased the *c*-Fos expression in nucleus accumbens (NAc), and suppressed the functional connectivity (FC) of NAc-dorsal raphe nucleus (DR) and NAc-cingulate cortex (CG). However, SCH58261 significantly shortened the duration of LOC induced by propofol, decreased the *c*-Fos expression in NAc, increased the *c*-Fos expression in DR, and elevated the FC of NAc-DR and NAc-CG. Collectively, our findings demonstrate the important roles played by $A_{2A}R$ in the LOC induced by propofol and suggest that the neural circuit between NAc-DR maybe controlled by $A_{2A}R$ in the mechanism of anesthesia induced by propofol.

Keywords: Loss of consciousness (LOC); Propofol; Adenosine A_{2A} receptor ($A_{2A}R$); Resting-state functional magnetic resonance imaging (rsfMRI); *c*-Fos

Abbreviations: ATP, adenosine triphosphate; $A_{2A}R$, adenosine A_{2A} receptor; BF, basal forebrain; BOLD, blood-oxygenation-level dependent; CBF, cerebral blood flow; CG, cingulate cortex; DR, dorsal raphe nucleus; FC, functional connectivity; fMRI, functional magnetic resonance imaging; GPCR, G protein-coupled receptors; GP, globus pallidus; IEG, immediate early gene; LOC, loss of consciousness; LORR, loss of righting reflex; NAc, nucleus accumbens; NREM, non-rapid eye movements; OB, olfactory bulb; PAG, periaqueductal gray; RRID, Research Resource Identifier (see scicrunch.org); ROI, regions of interest; RORR, recovery of righting reflex; RSD, retrosplenial dysgranular cortex; rsfMRI, resting-state functional magnetic resonance imaging; SE-EPI, spin-echo planar imaging sequence; STR, striatum; VTA, ventral tegmental area.

1 Introduction

General anesthesia, which has been used in clinical practice for about 170 years (Brandt & Artmeier-Brandt 2016), is a drug-induced reversible state, which includes unconsciousness, amnesia, analgesia, and immobility (Brown *et al.* 2010; Li *et al.* 2018c). Recovery from general anesthesia is still considered a passive process, depending on the elimination of anesthetic drug pharmacokinetics (Chemali *et al.* 2012). Many serious complications, including delayed recovery, agitation, delirium, and respiratory tract obstruction, may occur during recovery from general anesthesia (Li *et al.* 2018a). Delayed recovery is one of the most common complications (Alkire *et al.* 2007), and how to shorten the recovery time is a serious problem in clinical anesthesia. On the occasion of the 125th anniversary of the publication of *Science*, the question of how general anesthetics induce loss of consciousness (LOC) was posed (Kennedy & Norman 2005), but the mechanism of general anesthesia is not yet well understood (Bademosi *et al.* 2018), which is the reason for restricting the acceleration of recovery from general anesthesia. For more than 30 years, propofol, an intravenous anesthetic, has been commonly used for induction and maintenance of general anesthesia (Parks *et al.* 2016), but the mechanism of action is also still unknown and requires further investigation. Thus, propofol was selected as the starting point of general anesthesia research, which has great significance and importance to clinical practice.

In the past century, several sleep-related substances have been identified, including cytokines (Krueger *et al.* 1984), adenosine (Porkka-Heiskanen *et al.* 1997), urotensin II peptide (Huitron-Resendiz *et al.* 2005) and the anandamide prostaglandin D2 (Qu *et al.* 2006). Both neurons and glial cells can release adenosine, which is a metabolite mainly produced from adenosine triphosphate (ATP). Adenosine receptors (namely the A_1, A_{2A}, A_{2B} and A_3Rs) belong to the superfamily of G

protein-coupled receptors (GPCR) (Sheth *et al.* 2014), of which the A_{2A} receptor ($A_{2A}R$) has strong affinity for adenosine and is mainly distributed in brain regions such as striatum (STR), nucleus accumbens (NAc), globus pallidus (GP), and olfactory bulb (OB) (Fredholm *et al.* 2011; Fredholm *et al.* 2007; Ribeiro *et al.* 2002). In addition to increased physiological sleep (Porkka-Heiskanen *et al.* 1997), adenosine, as a neuromodulator, can enhance anesthetic potency (Kaputlu *et al.* 1998). The $A_{2A}R$ has been demonstrated to play a crucial role in the regulation of the physiological sleep process (Zhang *et al.* 2013; Hong *et al.* 2005), but its role in the LOC induced by general anesthesia is still unclear. Although general anesthesia-induced LOC is not the same as physiological sleep, the responsiveness to external stimuli and brain arousal systems are similarly decreased (Nelson *et al.* 2004; Alkire *et al.* 2008). Moreover, general anesthesia-induced LOC and physiological sleep have many similarities in terms of brain function, including brain electrical activity and brain metabolic activity (Sleigh *et al.* 1999; Alkire *et al.* 1999), therefore we speculated that $A_{2A}R$ may be able to regulate the LOC induced by general anesthetics.

As a noninvasive and unbiased analysis technique, functional magnetic resonance imaging (fMRI) has been widely used to investigate functional brain networks (Biswal *et al.* 1995; Zhong *et al.* 2019). Resting-state fMRI (rsfMRI), as a research hotspot in the field of fMRI, measures functional connectivity (FC) across brain regions by detecting temporal correlations of blood-oxygenation-level dependent (BOLD) signals (Liang *et al.* 2012), which indirectly reflects neural activity. With this technique, resting-state functional connectivity (rsFC) was found in different arousal states, such as awake, physiological sleep and anesthesia (Nallasamy & Tsao 2011; Paasonen *et al.* 2018). Additionally, rsfMRI is widely used in many neurodegenerative diseases, including Alzheimer's disease, dementia, schizophrenia and multiple sclerosis (van den Heuvel & Hulshoff Pol 2010;

Zhang *et al.* 2018). In summary, rsFC is strongly suggested to play a vital role in brain function. Furthermore, *c*-Fos, as an immediate early gene (IEG) with activity-dependent protein expression, is used as a marker of stimulus-induced neuronal activation (Lin *et al.* 2018; Marques-Carneiro *et al.* 2017), and the mapping of *c*-Fos expression in response to drug administration is one of the most suitable methods used to examine the response of specific brain regions with respect to the potential effects of drugs, including antipsychotics (Cohen *et al.* 2003; Sumner *et al.* 2004) and anesthetics (Yu *et al.* 2019).

With the help of the highly selective $A_{2A}R$ agonist and antagonist, the current study inspired us to investigate the role of $A_{2A}R$ in the LOC induced by propofol using observations of animal behaviors, rsfMRI and *c*-Fos staining to guide further research direction about the mechanism of general anesthesia. Thus, the current study could provide insights into the mechanisms of propofol and even other general anesthetics.

2 Materials and methods

2.1 Animals and housing conditions

The experimental protocol was approved by the Animal Ethics Committee of Zhongnan Hospital of Wuhan University (Ethics approval number: 02518103C), and all experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Adult female Sprague Dawley rats (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) (3 months old, weighing 240-300g) (RRID: RGD_10395233) were used in the current study. The animals were group-housed three per cage on a 12h light/dark cycle in a temperature-controlled (25±2˚C) room with free access to water and food, and the animals were

allowed to acclimatize to the environment for a week before the experiment commenced according to the standards established by the experimental animal laboratory at Zhongnan Hospital of Wuhan University. Every effort was made to minimize the number of animals used as well as pain and discomfort (*e.g.* Transcardial perfusion and tail vein catheterization were performed under isoflurane anesthesia to relieve the rat's pain).

2.2 Preparation and Delivery of Drugs

A total of forty female rats were divided into five different groups (n=8 per group): CGS (2.5mg/kg) group, *SCH*-3 (3mg/kg) group, SCH-6 (6mg/kg) group and SCH-8 (8mg/kg) group and vehicle group, and the selected doses of drugs were based on previous studies (El Yacoubi *et al.* 2000). There was no significant difference in body weight between the groups (p >0.05, Fig. S1), and the protocol for this experiment is depicted in Fig. 1.

Drugs treatment protocol: According to the previous report, $A_{2A}R$ highly selective agonist CGS21680 (CGS) (Selleckchem, Houston, TX, USA, cat.no. S2153) (2-p-(2-Carboxyethyl) phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride hydrate) and $A_{2A}R$ highly selective antagonist SCH58261 (SCH) (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) (Selleckchem, Houston, TX, USA, cat.no. S8104) were dissolved in dimethyl sulfoxide (DMSO) (MilliporeSigma, Burlington, MA, USA, cat.no. D2650) and then diluted in Cremophor EL (MilliporeSigma, Burlington, MA, USA, cat.no. 238470) and 0.9% NaCl (Final concentration: 15% DMSO and 15% Crempphor EL). Normal saline containing the same concentration of DMSO and Cremophor EL was served as the vehicle group. Drug solutions were prepared daily and administered via an intraperitoneal injection (*i.p.*) with a volume of 10 ml/kg.

Administration of anesthetics: The animal was initially anesthetized with isoflurane (RWD life science Co., Ltd., Shenzhen, China) (2%-4%) in oxygen, and tail vein catheterization was achieved with a single 24-gauge needle (Kangning medical equipment co., Ltd., Sichuan, China) connected to PE50 tubing and syringe, and the needle part was finally fixed onto the tail using tape. After catheterization, rats were allowed to fully recover from isoflurane in room air for at least 10 min based on previous research (Chemali *et al.* 2012). At the end, a bolus dose of propofol (15mg/kg) was delivered within 20 s through tail vein catheterization.

2.3 Animal behavior study - Time to recovery of the righting reflex after propofol bolus

A time-line diagram of the research design is illustrated in Fig. 1(A). Following previous protocol (Font *et al.* 2008), a bolus dose of CGS, SCH or vehicle was injected (*i.p.*) into the animals before propofol administration. After 15 min, propofol (Parks *et al.* 2016) (15mg/kg, Diprivan; AstraZeneca, UK) was administered via the lateral tail vein, and loss of righting reflex (LORR) was measured by rolling the rat onto its back and observing whether the animal was able to right itself. All rats were gently placed in the supine position on a heating pad (to maintain body temperature ~37° C) after losing righting reflex (measured from the start of the 20s injection). The time to achieve recovery of righting reflex (RORR) was recorded and defined as the period from the loss of the righting reflex until restoration of the righting reflex (*i.e.*, all four paws touching the floor).

2.4 Magnetic resonance imaging (MRI)

A time-line diagram of the research design is shown in Fig. 1(B). Based on the results of the behavioral study, the optimal dose of SCH was obtained. Then, a new group of rats was divided into three experimental groups (Total twenty-four female rats, n=8 per group) for the MRI study: vehicle group, CGS group and SCH-6 group.

The procedures of pre-experimental preparation for the fMRI study were similar to our previous studies (Li *et al.* 2014). The rat was initially anesthetized with isoflurane (4%-5%), and tail vein catheterization was performed as described above. Then, the anesthetized rat was placed in a 7.0 Tesla Biospec small animal magnetic resonance imaging system (Bruker, Ettlingen, Germany) under isoflurane anesthesia (1%-1.5%), and the body temperature was maintained with a heating pad. The rat's position was adjusted to the best position and securely fixed with the stereotaxic ear bar and bite bar. Physiological states of the animal were recorded with a series of equipment, such as a small animal monitoring system (Model 1025, Small Animal Instruments Inc., New York, NY, USA) and Dräger Infinity Delta monitor, including a rectal temperature probe, respiration pneumatic sensor, arterial pressure sensor and cardiogram electrodes. Arterial blood samples were collected from the femoral artery and analyzed (i-STAT Model 300, Abbott Point of Care Inc., Princeton, NJ, USA) for blood gas measurements, such as pH, pCO_2, pO_2 and blood glucose values. The drugs (CGS, SCH and vehicle) were administered i.p. After 15 min, propofol was intravenously injected through the tail vein and the isoflurane stopped. The magnetic resonance imaging data was collected.

All MRI experiments were performed on a 7.0T Bruker Biospec70/20 USR small animal MR system (Bruker, Germany) with a 10mm surface coil. Resting-state fMRI scans were acquired using single-shot spin-echo planar imaging sequence (SE-EPI). Each scan consisted of 300 volumes obtained by the following parameters: TR = 1000 ms, TE =14 ms, flip angle = 45◦, FOV = 20 mm×20 mm, spatial resolution = 0.31 mm×0.31 mm, slice thickness = 1 mm, and 20 slices in total. An additional T2-weighted anatomical scan was acquired with the same geometry as the fMRI scan

using the following parameters: TR = 5000 ms, effective TE = 12 ms, RARE factor = 8, matrix = 256×256, spatial resolution = 0.08 mm×0.08 mm.

2.5 Immunohistochemistry of *c*-Fos

Two hours after treatment with drugs and propofol, the rat was deeply anesthetized with an overdose of isoflurane and transcardially perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. The rat brain was removed from the skull, and post-fixed with 4% paraformaldehyde at 4°C. The fixed brain was sectioned into coronal slices with 40μm thickness using a cryostat microtome (Thermo Fisher, NX50, Waltham, MA).

Free-floating brain slices were initially washed with PBS (three times, 8 min each). The slices were rinsed and transferred to the blocking buffer (10% normal goat serum, 0.3% Triton X-100 in PBS) for 1 h at 37°C. After the blocking procedure, the slices were incubated (72 h, 4°C) with anti-*c*-Fos rabbit polyclonal antibodies (1:1000; Cell Signaling, cat.no. 2250, RRID: AB_2247211). Antibodies were diluted with 3% normal goat serum, 0.1% Triton X-100 in PBS. After washing in PBS (3 times, 10 min each), the slices were further incubated with a FITC-labeled goat anti-rabbit IgG (1:200, 2h; Abcam, UK, cat.no. ab6717, RRID: AB_955238) for 2h at 37°C, stained with DAPI for 10 min at room temperature, washed with PBS (3 times, 10 min each), and mounted in 70% glycerol. Finally, the immunostained brain slice was imaged using a virtual microscopy slide-scanning system (Olympus, VS 120, Tokyo, Japan). Images of brain slices containing the region of interest (ROI) were cropped out and counted using ImageJ (National Institutes of Health, Bethesda, MD).

2.6 Data processing

All fMRI data was processed with Statistical Parametric Mapping 12 (SPM 12, http://www.fil.ion.ucl.ac.uk/spm/) in MATLAB (R2018b, Mathworks Inc. 2018) software for time segmentation and rearrangement, head movement correction, spatial standardization, smoothing, *etc*. After processing, the extracranial tissue was removed and the brain tissue retained. For analysis of the resting state fMRI, the preprocessed fMRI data was detrended, covariant regressed and filtered (0.01-0.1Hz) using DPABI. A series of brain regions of interest (ROI) were automatically defined with the rat brain anatomic atlas (Nie *et al.* 2013), which are listed in the legends of Fig. 2. The rsFCs were computed using Pearson correlation, and Fisher z-transformation was performed before statistical analysis. For analysis of ROI-voxel correlation, the ROIs were automatically defined with the atlas (Nie *et al.* 2013). The DPABI software in MATLAB was used to calculate the functional connection diagrams, and the differences were analyzed with single-sample *t*-test within groups (FDR, $p<0.05$) and two-sample *t*-test between groups ($p<0.05$).

2.7 Statistical Analysis

For the whole experiment, the experimenter was unaware of the group allocation of each animal during the experimental procedures and statistical analysis. The statistical analyses were performed with SPSS 24.0 (IBM, New York, USA), GraphPad Prism 6.0 (GraphPad, New York, USA) and MATLAB (R2018b, Mathworks Inc. 2018). Shapiro-Wilk test was used to analyze the normality of data, and we found that the data was normally distributed. All data was expressed as the means ± standard error of the mean (SEM). Student's *t*-test was used to compare the differences between the two groups and one-way analysis of variance (ANOVA) was used for more than two groups, followed by a Student-Newman-Keuls multiple range test for post hoc comparisons. A *p* value less

than 0.05 was considered statistically significant. In this study, no randomization methods were used to allocate samples. No pre-registration, sample calculation, exclusion criteria, or testing for outliers was performed in this study. The study was exploratory and no primary and secondary endpoints were pre-specified.

3 Results

3.1 Effects of CGS and SCH for propofol anesthesia

The effects of CGS and different doses of SCH on LORR and RORR of animals after propofol anesthesia are illustrated in Fig. 2. Results show that the time taken to achieve RORR of rats which only received the vehicle was 905 ± 85s. Compared to the vehicle group, CGS significantly prolonged the period to 1435 ± 85s ($p<0.001$, Fig. 2A), however, SCH-6 and SCH-8 significantly shortened the time to 538 ± 33s and 569 ± 30s, respectively ($p<0.001$, Fig. 2A), SCH-3 reduced the time (759 ± 50s) but there was no significant difference ($p=0.101$, Fig. 2A). However, there was no difference in the time of LORR between these groups ($p>0.05$， Fig. 2B). As a highly selective agonist (CGS) and antagonist (SCH) for $A_{2A}R$, results of the behavioral study indicate that $A_{2A}R$ could play a key role in LOC induced by propofol, and its mechanism deserves further study.

3.2 Resting state fMRI analysis for the whole brain functional connectivity

The physiology of anesthetized rats was carefully monitored and controlled. All measured physiological parameters are shown to be in the normal range (Table 1). Statistical comparisons show that there were no significant differences in the physiological parameters among the three groups.

The resting state fMRI method was utilized to analyze FC in the whole brain of the animals under different chemical treatments. The strength of FC between two different brain regions is illustrated by the color of every cell in the matrix (Fig. 3A-C). For the CGS group (Fig. 3A), 14 pairs of FC in brain regions were elevated and 25 pairs suppressed, compared to the vehicle group (Fig. 3B). In the SCH-6 group (Fig 3C), 12 pairs were elevated and only 1 pair suppressed (correlation between prelimbic cortex and insular cortex) compared to the vehicle group (Fig. 3B).

The correlations of FCs in the brain under different drug treatments were collected and illustrated (Fig. 3D–F). As shown in Fig. 3D, 247 pairs were above the central line ($y=x$) and 131 pairs below it (R^2=0.8158). The results indicate that CGS suppressed FC between brain regions compared with the vehicle group. In Fig. 3E, 126 pairs were above the central line ($y=x$) and 252 pairs below it (R^2=0.8854), which means that SCH-6 elevated FC between brain regions compared to the vehicle group. Compared with CGS (Fig. 3F), SCH-6 significantly elevated FC between brain regions (288 points above the central line and 90 points below it, R^2=0.7706). Taken together, these results suggest that there is an association between $A_{2A}R$ and FC of brain regions. However, it is necessary to further analyze these results in order to determine the most meaningful brain regions in the next stage of the research.

3.3 Functional connectivity of specific brain regions

The basal forebrain (BF) plays key roles in controlling sleep and wakefulness, and includes the NAc one of the larger nucleus (Xu *et al.* 2015). Meanwhile, the $A_{2A}R$ is mainly distributed in STR and NAc, which were selected as the seed regions of interest (ROIs) and rsFC was analyzed between these brain regions. Thus, to evaluate changes in cerebral activity after treatment with CGS and SCH on the propofol anesthetized animals, the representative seed-based connectivity maps (STR

and NAc) were obtained from the vehicle group, and differences between the vehicle and CGS groups, vehicle and SCH-6 groups are presented in Fig. 4.

Consistent with results of the whole brain rsfMRI, the values of FCs in the CGS group were suppressed, including the NAc-cingulate cortex (CG), NAc-dorsal raphe nucleus (DR), NAc-periaqueductal gray (PAG) and STR-retrosplenial dysgranular cortex (RSD), except the STR-hypothalamus. For the SCH-6 group, FC values of NAc-CG, NAc-DR, NAc-PAG, STR-cortex, STR-thalamus and STR-hypothalamus were elevated compared to the vehicle group. Taken together, these different connections suggest that $A_{2A}R$ affects propofol induced LOC by altering FCs between brain regions, such as NAc-CG, NAc-DR and NAc-PAG. Furthermore, Liang and his colleagues found that the negative rsFC might serve as an important biomarker to help evaluate the functionality of neural circuits by studying the rsFC between the infralimbic cortex and amygdala (Liang *et al.* 2012). Thus, these three common statistically different neural circuits have important research significance for the next stage.

3.4 *C*-Fos expression

C-Fos is always used as a marker of neuronal activation (Lin *et al.* 2018). Results show that CGS increased the expression of *c*-Fos in NAc, while SCH-6 reduced the expression of *c*-Fos compared to that of pretreatment with vehicle ($p < 0.05$, Fig. 5A-J). For another region DR, SCH-6 increased the expression of *c*-Fos ($p < 0.05$, Fig. 6A-J), while CGS had no effect on the expression of *c*-Fos compared to that of pretreatment with vehicle ($p > 0.05$, Fig. 6A-J). Furthermore, CGS and SCH-6 had no effect on the *c*-Fos expression in neither CG ($p > 0.05$, Supplemental information Fig. 2A-J) nor STR ($p > 0.05$, Supplemental information Fig. 3A-J). Taken together, these results suggest that neuronal activities in NAc and DR could play important roles in propofol induced LOC.

4 Discussion

The aim of this study was to investigate the important roles played by $A_{2A}R$ in propofol induced LOC in rats using animal behaviors, rsfMRI and *c*-Fos immunofluorescence staining to screen the relevant brain regions under propofol general anesthesia. Thereby, it could provide an avenue for future research on the mechanism of anesthesia induced by propofol.

Results show that $A_{2A}R$ agonist CGS significantly prolonged the duration of LOC induced by propofol, while $A_{2A}R$ antagonist SCH shortened it. Moreover, CGS increased *c*-Fos expression in NAc, while SCH decreased *c*-Fos expression in NAc and increased *c*-Fos expression in DR. Furthermore, we also found that CGS suppressed FC between brain regions, while SCH elevated FC in the brain. Compared with the vehicle group, FC of NAc-CG and NAc-DR were common brain regions with statistical differences in FC of the brain regions of the animals among CGS and SCH-3 groups. Taken together, these results indicate the important role of $A_{2A}R$ in propofol induced LOC and furthermore, the NAc-CG and NAc-DR neural circuit may be worth of future study to examine the mechanism of propofol induced anesthesia. To the best of our knowledge, this is the first report to explore the influence of $A_{2A}R$ on LOC induced by propofol using rsfMRI to identify the relevant brain regions.

As a neural sleep factor, adenosine has been widely studied for its contribution to sleep-wakefulness states (Porkka-Heiskanen *et al.* 1997; Porkka-Heiskanen *et al.* 2000; Halassa *et al.* 2009; Lazarus *et al.* 2019). During spontaneous sleep-wake cycles, it has been reported that adenosine levels collected from cats using *in vivo* microdialysis were higher during non-rapid eye movement (non-REM, NREM) sleep compared to wakefulness in several brain regions, such as the

BF, thalamus and DR (Porkka-Heiskanen *et al.* 1997; Porkka-Heiskanen *et al.* 2000). Extracellular studies showed that adenosine reacts with one of four adenosine receptors, including A_1, A_{2A}, A_{2B}, and A_3Rs (Fredholm *et al.* 2011; Fredholm *et al.* 2001). In contrast to A_{2B}R and A_3R, the expression of A_1R and A_{2A}R appears to be higher, thus these two receptor types could be primarily involved in the sleep-wakefulness regulation (Fredholm *et al.* 2005). Physical sleep and anesthesia are almost similar on many fronts, including the responsiveness to external stimuli (Nelson *et al.* 2004), brain electrical activity (Sleigh *et al.* 1999) and metabolic activity (Alkire *et al.* 1999). Additionally, adenosine has been reported to have the ability to shorten the onset time and extend the duration of propofol (Kaputlu *et al.* 1998), similarly the A_1 receptor agonist (N6-p-sulfophenyladenosine) has been shown to increase the time needed to recover from halothane anesthesia (Tanase *et al.* 2003). However, the role of A_{2A}R in affecting general anesthesia and its mechanism remain unclear. As one of the most commonly used intravenous anesthetic in clinical practice, propofol was the target for research in the present study. Results showed that the A_{2A}R agonist prolonged the duration of propofol induced LOC, while the A_{2A}R antagonist shortened it. These observations are in agreement with the abovementioned studies, which indicated the involvement of A_{2A}R in the regulation of propofol. However, the mechanism by which the A_{2A}R agonist prolonged the LOC induced by propofol remains to be further investigated.

As a research hotspot in the field of fMRI and a noninvasive technique, rsfMRI has been widely used for assessing FC between different brain regions, identifying functional circuits, and exploring the changes in brain functional networks in both humans and animals under different conditions, including unconsciousness induced by general anesthetics (Biswal *et al.* 1995; Paasonen *et al.* 2018; Li *et al.* 2018b; Wang *et al.* 2013). An earlier human study indicated that FC between the cortex

and subcortical centers significantly decreased under propofol sedation compared to sleep (Li *et al.* 2018b). In addition, results from male adult squirrel monkeys showed that isoflurane decreased the inter-voxel connectivity around seed regions and weakened inter-regional FC across all pairs of ROIs (Wu *et al.* 2016). Results from the above studies suggest that the LOC induced by anesthetics could be related to the decline of brain functional connectivity. In the present study, we found that $A_{2A}R$ agonist prolonged the LOC induced by propofol, thus the rsfMRI was utilized to investigate whether there were any changes involved in brain FC. In addition, it is now well established from a variety of studies, that $A_{2A}R$ are expressed with the greatest abundance in regions of STR and NAc, which were selected as seed ROIs to analyze FC between the brain regions.

Results of our study showed that $A_{2A}R$ agonist-CGS suppressed the FC of NAc-DR and NAc-CG, while $A_{2A}R$ antagonist-SCH elevated their FC. Therefore, the changes of FC in NAc-DR and NAc-CG could play important roles in the LOC induced by propofol. It is worth noting that rsfMRI was used to measure functional connectivity across brain regions by detecting the correlations of BOLD signals (Liang *et al.* 2012), and both animal and human studies have demonstrated that $A_{2A}R$ antagonist/agonist exposure leads to constriction/expansion of cerebral vessels and reduced/increased cerebral blood flow (CBF) in mainly the $A_{2A}R$ distributed region (Pelligrino *et al.* 2010; Ngai *et al.* 2001). FC is defined as the correlation between two brain regions to the same time dimension, and $A_{2A}R$ antagonist/agonist altered CBF in the brain region where the receptors are distributed, and FC of this region with the other brain regions should be decreased due to the altered brain vasculature. However, the changes in FC for agonist/antagonist were opposite in the current study, thus the other influencing factors of FC - neuronal activities could play major roles in changing the FC in these brain regions, which were ascertained by the results of *c*-Fos.

C-Fos is used to reflect the activation of neurons (Lin *et al.* 2018). Previous findings show that perfusing CGS into the subarachnoid space just anterior to the ventrolateral preoptic area increased NREM and rapid eye movement (REM) sleep with a significant increase in *c*-Fos expression in NAc (Satoh *et al.* 1999; Scammell *et al.* 2001). In the present study, CGS also significantly increased the expression of *c*-Fos in NAc, while SCH-6 decreased the expression. These observations are in line with the above mentioned studies, which indicated that $A_{2A}R$ could modulate the activities of neurons in NAc, thereby affecting the LOC induced by propofol. The region of NAc located in the ventral striatum, is one of the forebrain nuclei playing a major role in the sleep-wake cycle control (Lazarus *et al.* 2012), possibly by inhibiting wake-promoting nuclei in the brainstem and the hypothalamus (Sardi *et al.* 2018). The DR, located in the brainstem and containing almost 50% of serotonergic (5-HT) neurons in the brain (Zhang *et al.* 2012), is involved in several functions, including sleep, temperature regulation, stress responses, and anxiety behaviors (Hernandez-Vazquez *et al.* 2019). A study by Cui and colleagues showed that the application of $CaCl_2$ significantly increased *c*-Fos expression of 5-HT neurons in DR by promoting waking in rats (Cui *et al.* 2016). Results from recent research indicate that isoflurane anesthesia inhibited 5-HT neuronal activity, as illustrated by the decrease in the number of *c*-Fos-immunoreactive serotonergic neurons compared to the control group (Yang *et al.* 2019). In our study, compared to the vehicle group, $A_{2A}R$ antagonist shortened the duration of propofol-induced LOC by significantly increasing *c*-Fos expression in DR. These results are in agreement with the abovementioned studies indicating that neurons in DR play an important role during sleep wakefulness procedures. $A_{2A}R$ agonist prolonged the duration of unconsciousness induced by propofol, but there was no statistical difference in *c*-Fos expression compared to the vehicle group. These results are interesting and explainable. A

possible explanation for this might be the administration of a larger induction dose of propofol (15mg/kg, IV), which severely suppressed the expression of *c*-Fos in the vehicle group (as shown in Fig. 5). Although CGS prolonged the time of LOC induced by propofol, the expression of *c*-Fos cannot be further reduced due to the presence of the "floor effect".

NAc consists of two types of neurons, including GABAergic projection neurons and interneurons, of which GABAergic projection neurons are divided into enkephalinergic and dynorphinergic neurons. $A_{2A}R$ is mainly expressed in GABAergic enkephalinergic neurons and associated with the indirect efferent pathway of the basal ganglia system (Schiffmann *et al.* 2007; Ferre *et al.* 2007). Therefore, the projected target region of NAc neurons does not only represent the region projected by NAc $A_{2A}R$ neurons. As Cre-LoxP technology is widely used to trace specific types of neurons, projected brain regions of $A_{2A}R$ neurons in NAc were tracked, including STR, ventral tegmental area (VTA) and DR (Zhang *et al.* 2013). It is worth noting that the projection between NAc and DR is not to a single direction. Muzerelle and colleagues confirmed that 5-HT neurons in the DR could project to multiple brain regions, such as the hypothalamus, STR, VTA and NAc (Muzerelle *et al.* 2016). Based on the above-mentioned research and the results of the rsfMRI and *c*-Fos staining in the current study, the connection or interaction between NAc and DR may play a key role in the LOC induced by propofol and is worthy of further research.

Several limitations are worth noting. First, female rats were only utilized as experimental subjects based on the latest research results (Shansky 2019), but the effect of sex on results is not clear and is worth studying in the future. Second, we did not use electroencephalogram (EEG) to monitor the level of consciousness of rats in the experiment due to the limited experimental conditions. Third, neuronal activities were detected with *c*-Fos expression in different brain regions, and the type of

neurons should, therefore, be identified in future. Fourth, although CGS21680 has been introduced as a selective and potent adenosine A_2 receptor agonist with approximately 140-fold selectivity for A_2 over A_1 receptors in the rat brain (Hutchison *et al.* 1989), its effect on the A_1 receptor does exist. Moreover, A_1R activation causes profound sedation (Dunwiddie & Worth 1982) and increases the duration of LOC induced by halothane anesthesia (Tanase *et al.* 2003). In this study, the effect of A_1R activation induced by CGS21680 could not be completely excluded. Thus, the difference in activation of A_1R and A_{2A}R induced by CGS 21680 and the duration of propofol-induced LOC should be further investigated.

5 Conclusion

The present study was designed to determine the effects of A_{2A}R on propofol induced LOC and the brain regions that may be involved. The results of this investigation show that A_{2A}R agonist inhibited FC between NAc and DR, increased *c*-Fos expression in NAc, and prolonged the duration of unconsciousness induced by propofol, while the A_{2A}R antagonist had the opposite effect. These findings have significant implications for our understanding of the role played by A_{2A}R in propofol induced LOC. Considerably more work will need to be done in order to determine whether neurons in NAc and DR or their connectivity are involved in propofol induced LOC or even general anesthetic.

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Conflict of interest

The authors declare no competing financial interests.

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Figure legends

Fig. 1 A time-line diagram of the research design. (A) Seven days' following habituation, tail vein catheterization was performed under isoflurane anesthesia. 10 minutes later, rats were injected with vehicle, SCH or CGS (*i.p.*). After 15 minutes, rats were given propofol (*i.v.*), followed by recording the duration of LORR and RORR. (B) RsfMRI scanning and physiological monitoring were performed after the propofol injection, and *c*-Fos staining was executed 2 hours after vehicle, SCH or CGS administration.

Fig. 2 CGS increased the time of RORR, while SCH decreased it. Comparisons of RORR (A) and LORR (B) of rats in different groups – CGS (2.5mg/kg), SCH-3 (3mg/kg), SCH-6 (6mg/kg), SCH-8 (8mg/kg) and vehicle groups (n= 8 per group). "n" indicates the number of rats included. Note: The data is plotted as the mean ± standard error of the mean for each group, $^{***}p<0.001$ vs vehicle group.

Fig. 3 The FC matrices in the whole brain for different groups (A-C) and the scatter plots of FC between two different groups (D-F). Note: A-CGS; B-vehicle group; C-SCH-6; # or *: elevated or suppressed FC values compared with FC in the vehicle group (t-test, $p < 0.05$, false discovery rate corrected, n= 8 per group). "n" indicates the number of rats included. Abbreviations of brain regions-Hb, Habenula nucleus; NAc, nucleus accumbens; Str, striatum; Tu, tubercle olfactory; Prc, prelimbic cortex; CG, cingulate cortex; PiC, piriform cortex; SC, somatosensory cortex; AUC, auditory cortex; DB, diagonal band; AM, amygdala; SP, septum; IC, insular cortex; SN, substantia nigra; PO, preoptic nucleus; VTA, ventral tegmental area; PrhC, perirhinal cortex; Ect, ectorhinal cortex; EnC, entorhinal cortex; TeAC, temporal association cortex; SIC, superior and inferior colliculus; VC, visual cortex; RC, retrosplential cortex; MC, motor cortex; RE, reticular nucleus; DR, dorsal raphe nucleus; HTh, hypothalamus; ThM, thalamus.

Fig. 4 The whole brain functional connectivity of specific brain regions (NAc and STR). Note: Maps of correlation coefficients are overlaid on T2-weighted anatomic images; the statistical comparison was performed with voxel-wise t-tests ($p<0.05$ with false discovery rate correction，n= 8 per group). "n" indicates the number of rats included. Distances to Bregma (mm) are labeled at the bottom of each image. The larger |t| indicates more variation.

Fig. 5 Effects of CGS/SCH-6 on c-Fos expression in NAc (from bregma 1.8mm). Note: A-I: Representative microphotographs of c-Fos expression (green) and DAPI (blue) immunofluorescence staining in NAc; B–C, E–F, and H–I: the magnified images of the boxed areas in A, D, and G, respectively, and scale bar=200 μm. J: Statistical analysis of c-Fos expression in NAc (Mean ± S.E.; n=3 per group). "n" indicates the number of animals included. NAc, nucleus accumbens; $^{*}p<0.05$ vs vehicle group; LV, Lateral ventricles.

Fig. 6 Effects of CGS/SCH-6 on c-Fos expression in DR (Dorsal raphe nucleus, from bregma 7.92 mm). Note: A-I: Representative microphotographs of c-Fos expression (green) and DAPI (blue) immunofluorescence staining in DR; B–C, E–F, and H–I: magnified images of the boxed areas in A, D, and G, respectively, and scale bar=200 μm. J: Statistical analysis of c-Fos expression

in DR (Mean ± S.E.; n=3 per group). “n” indicates the number of animals included. $^{*}p<0.05$ vs vehicle group; Aq: aqueduct.