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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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1 **Abstract**

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

18
19 **Keywords:** Loss of consciousness (LOC); Propofol; Adenosine A_{2A} receptor (A_{2A}R); Resting-state
20 functional magnetic resonance imaging (rsfMRI); *c*-Fos

21

22 **Abbreviations:** ATP, adenosine triphosphate; A_{2A}R, adenosine A_{2A} receptor; BF, basal forebrain;
23 BOLD, blood-oxygenation-level dependent; CBF, cerebral blood flow; CG, cingulate cortex; DR,
24 dorsal raphe nucleus; FC, functional connectivity; fMRI, functional magnetic resonance imaging;
25 GPCR, G protein-coupled receptors; GP, globus pallidus; IEG, immediate early gene; LOC, loss of
26 consciousness; LORR, loss of righting reflex; NAc, nucleus accumbens; NREM, non-rapid eye
27 movements; OB, olfactory bulb; PAG, periaqueductal gray; RRID, Research Resource Identifier
28 (see scicrunch.org); ROI, regions of interest; RORR, recovery of righting reflex; RSD, retrosplenial
29 dysgranular cortex; rsfMRI, resting-state functional magnetic resonance imaging; SE-EPI, spin-
30 echo planar imaging sequence; STR, striatum; VTA, ventral tegmental area.
31

32 **1 Introduction**

33 General anesthesia, which has been used in clinical practice for about 170 years (Brandt & Artmeier-
34 Brandt 2016), is a drug-induced reversible state, which includes unconsciousness, amnesia,
35 analgesia, and immobility (Brown *et al.* 2010; Li *et al.* 2018c). Recovery from general anesthesia is
36 still considered a passive process, depending on the elimination of anesthetic drug pharmacokinetics
37 (Chemali *et al.* 2012). Many serious complications, including delayed recovery, agitation, delirium,
38 and respiratory tract obstruction, may occur during recovery from general anesthesia (Li *et al.*
39 2018a). Delayed recovery is one of the most common complications (Alkire *et al.* 2007), and how
40 to shorten the recovery time is a serious problem in clinical anesthesia. On the occasion of the 125th
41 anniversary of the publication of *Science*, the question of how general anesthetics induce loss of
42 consciousness (LOC) was posed (Kennedy & Norman 2005), but the mechanism of general
43 anesthesia is not yet well understood (Bademosi *et al.* 2018), which is the reason for restricting the
44 acceleration of recovery from general anesthesia. For more than 30 years, propofol, an intravenous
45 anesthetic, has been commonly used for induction and maintenance of general anesthesia (Parks *et*
46 *al.* 2016), but the mechanism of action is also still unknown and requires further investigation. Thus,
47 propofol was selected as the starting point of general anesthesia research, which has great
48 significance and importance to clinical practice.

49 In the past century, several sleep-related substances have been identified, including cytokines
50 (Krueger *et al.* 1984), adenosine (Porkka-Heiskanen *et al.* 1997), urotensin II peptide (Huitron-
51 Resendiz *et al.* 2005) and the anandamide prostaglandin D2 (Qu *et al.* 2006). Both neurons and glial
52 cells can release adenosine, which is a metabolite mainly produced from adenosine triphosphate
53 (ATP). Adenosine receptors (namely the A₁, A_{2A}, A_{2B} and A₃Rs) belong to the superfamily of G

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

67 As a noninvasive and unbiased analysis technique, functional magnetic resonance imaging (fMRI)
68 has been widely used to investigate functional brain networks (Biswal *et al.* 1995; Zhong *et al.*
69 2019). Resting-state fMRI (rsfMRI), as a research hotspot in the field of fMRI, measures functional
70 connectivity (FC) across brain regions by detecting temporal correlations of blood-oxygenation-
71 level dependent (BOLD) signals (Liang *et al.* 2012), which indirectly reflects neural activity. With
72 this technique, resting-state functional connectivity (rsFC) was found in different arousal states,
73 such as awake, physiological sleep and anesthesia (Nallasamy & Tsao 2011; Paasonen *et al.* 2018).
74 Additionally, rsfMRI is widely used in many neurodegenerative diseases, including Alzheimer's
75 disease, dementia, schizophrenia and multiple sclerosis (van den Heuvel & Hulshoff Pol 2010;

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

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

88

89 **2 Materials and methods**

90 **2.1 Animals and housing conditions**

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

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

103 **2.2 Preparation and Delivery of Drugs**

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

109 *Drugs treatment protocol:* According to the previous report, A_{2A}R highly selective agonist
110 CGS21680 (CGS) (Selleckchem, Houston, TX, USA, cat.no. S2153) (2-p-(2-Carboxyethyl)
111 phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride hydrate) and A_{2A}R highly
112 selective antagonist SCH58261 (SCH) (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-
113 1,2,4-triazolo[1,5-c]pyrimidine) (Selleckchem, Houston, TX, USA, cat.no. S8104) were dissolved
114 in dimethyl sulfoxide (DMSO) (MilliporeSigma, Burlington, MA, USA, cat.no. D2650) and then
115 diluted in Cremophor EL (MilliporeSigma, Burlington, MA, USA, cat.no. 238470) and 0.9% NaCl
116 (Final concentration: 15% DMSO and 15% Cremophor EL). Normal saline containing the same
117 concentration of DMSO and Cremophor EL was served as the vehicle group. Drug solutions were
118 prepared daily and administered via an intraperitoneal injection (*i.p.*) with a volume of 10 ml/kg.

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

126 **2.3 Animal behavior study - Time to recovery of the righting reflex after propofol bolus**

127 A time-line diagram of the research design is illustrated in Fig. 1(A). Following previous protocol
128 (Font *et al.* 2008), a bolus dose of CGS, SCH or vehicle was injected (*i.p.*) into the animals before
129 propofol administration. After 15 min, propofol (Parks *et al.* 2016) (15mg/kg, Diprivan;
130 AstraZeneca, UK) was administered via the lateral tail vein, and loss of righting reflex (LORR) was
131 measured by rolling the rat onto its back and observing whether the animal was able to right itself.
132 All rats were gently placed in the supine position on a heating pad (to maintain body temperature
133 ~37° C) after losing righting reflex (measured from the start of the 20s injection). The time to
134 achieve recovery of righting reflex (RORR) was recorded and defined as the period from the loss
135 of the righting reflex until restoration of the righting reflex (*i.e.*, all four paws touching the floor).

136 **2.4 Magnetic resonance imaging (MRI)**

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

141 The procedures of pre-experimental preparation for the fMRI study were similar to our previous
142 studies (Li *et al.* 2014). The rat was initially anesthetized with isoflurane (4%-5%), and tail vein
143 catheterization was performed as described above. Then, the anesthetized rat was placed in a 7.0
144 Tesla Biospec small animal magnetic resonance imaging system (Bruker, Ettlingen, Germany)
145 under isoflurane anesthesia (1%-1.5%), and the body temperature was maintained with a heating
146 pad. The rat's position was adjusted to the best position and securely fixed with the stereotaxic ear
147 bar and bite bar. Physiological states of the animal were recorded with a series of equipment, such
148 as a small animal monitoring system (Model 1025, Small Animal Instruments Inc., New York, NY,
149 USA) and Dräger Infinity Delta monitor, including a rectal temperature probe, respiration
150 pneumatic sensor, arterial pressure sensor and cardiogram electrodes. Arterial blood samples were
151 collected from the femoral artery and analyzed (i-STAT Model 300, Abbott Point of Care Inc.,
152 Princeton, NJ, USA) for blood gas measurements, such as pH, pCO₂, pO₂ and blood glucose values.
153 The drugs (CGS, SCH and vehicle) were administered i.p. After 15 min, propofol was intravenously
154 injected through the tail vein and the isoflurane stopped. The magnetic resonance imaging data was
155 collected.

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

162 using the following parameters: TR = 5000 ms, effective TE = 12 ms, RARE factor = 8, matrix =
163 256×256, spatial resolution = 0.08 mm×0.08 mm.

164 **2.5 Immunohistochemistry of *c-Fos***

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

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

182

183

184 **2.6 Data processing**

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

197 **2.7 Statistical Analysis**

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

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

210

211 **3 Results**

212 **3.1 Effects of CGS and SCH for propofol anesthesia**

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

222 **3.2 Resting state fMRI analysis for the whole brain functional connectivity**

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

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

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

243 **3.3 Functional connectivity of specific brain regions**

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

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

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

262 **3.4 C-Fos expression**

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

271

272 **4 Discussion**

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

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

288 As a neural sleep factor, adenosine has been widely studied for its contribution to sleep-
289 wakefulness states (Porkka-Heiskanen *et al.* 1997; Porkka-Heiskanen *et al.* 2000; Halassa *et al.*
290 2009; Lazarus *et al.* 2019). During spontaneous sleep-wake cycles, it has been reported that
291 adenosine levels collected from cats using *in vivo* microdialysis were higher during non-rapid eye
292 movement (non-REM, NREM) sleep compared to wakefulness in several brain regions, such as the

293 BF, thalamus and DR (Porkka-Heiskanen *et al.* 1997; Porkka-Heiskanen *et al.* 2000). Extracellular
294 studies showed that adenosine reacts with one of four adenosine receptors, including A₁, A_{2A}, A_{2B},
295 and A₃Rs (Fredholm *et al.* 2011; Fredholm *et al.* 2001). In contrast to A_{2B}R and A₃R, the expression
296 of A₁R and A_{2A}R appears to be higher, thus these two receptor types could be primarily involved in
297 the sleep-wakefulness regulation (Fredholm *et al.* 2005). Physical sleep and anesthesia are almost
298 similar on many fronts, including the responsiveness to external stimuli (Nelson *et al.* 2004), brain
299 electrical activity (Sleigh *et al.* 1999) and metabolic activity (Alkire *et al.* 1999). Additionally,
300 adenosine has been reported to have the ability to shorten the onset time and extend the duration of
301 propofol (Kaputlu *et al.* 1998), similarly the A₁ receptor agonist (N⁶-p-sulfophenyladenosine) has
302 been shown to increase the time needed to recover from halothane anesthesia (Tanase *et al.* 2003).
303 However, the role of A_{2A}R in affecting general anesthesia and its mechanism remain unclear. As
304 one of the most commonly used intravenous anesthetic in clinical practice, propofol was the target
305 for research in the present study. Results showed that the A_{2A}R agonist prolonged the duration of
306 propofol induced LOC, while the A_{2A}R antagonist shortened it. These observations are in agreement
307 with the abovementioned studies, which indicated the involvement of A_{2A}R in the regulation of
308 propofol. However, the mechanism by which the A_{2A}R agonist prolonged the LOC induced by
309 propofol remains to be further investigated.

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

315 and subcortical centers significantly decreased under propofol sedation compared to sleep (Li *et al.*
316 2018b). In addition, results from male adult squirrel monkeys showed that isoflurane decreased the
317 inter-voxel connectivity around seed regions and weakened inter-regional FC across all pairs of
318 ROIs (Wu *et al.* 2016). Results from the above studies suggest that the LOC induced by anesthetics
319 could be related to the decline of brain functional connectivity. In the present study, we found that
320 A_{2A}R agonist prolonged the LOC induced by propofol, thus the rsfMRI was utilized to investigate
321 whether there were any changes involved in brain FC. In addition, it is now well established from a
322 variety of studies, that A_{2A}R are expressed with the greatest abundance in regions of STR and NAc,
323 which were selected as seed ROIs to analyze FC between the brain regions.

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

337 *C-Fos* is used to reflect the activation of neurons (Lin *et al.* 2018). Previous findings show that
338 perfusing CGS into the subarachnoid space just anterior to the ventrolateral preoptic area increased
339 NREM and rapid eye movement (REM) sleep with a significant increase in *c-Fos* expression in NAc
340 (Satoh *et al.* 1999; Scammell *et al.* 2001). In the present study, CGS also significantly increased the
341 expression of *c-Fos* in NAc, while SCH-6 decreased the expression. These observations are in line
342 with the above mentioned studies, which indicated that A_{2A}R could modulate the activities of
343 neurons in NAc, thereby affecting the LOC induced by propofol. The region of NAc located in the
344 ventral striatum, is one of the forebrain nuclei playing a major role in the sleep-wake cycle control
345 (Lazarus *et al.* 2012), possibly by inhibiting wake-promoting nuclei in the brainstem and the
346 hypothalamus (Sardi *et al.* 2018). The DR, located in the brainstem and containing almost 50% of
347 serotonergic (5-HT) neurons in the brain (Zhang *et al.* 2012), is involved in several functions,
348 including sleep, temperature regulation, stress responses, and anxiety behaviors (Hernandez-
349 Vazquez *et al.* 2019). A study by Cui and colleagues showed that the application of CaCl₂
350 significantly increased *c-Fos* expression of 5-HT neurons in DR by promoting waking in rats (Cui
351 *et al.* 2016). Results from recent research indicate that isoflurane anesthesia inhibited 5-HT neuronal
352 activity, as illustrated by the decrease in the number of *c-Fos*-immunoreactive serotonergic neurons
353 compared to the control group (Yang *et al.* 2019). In our study, compared to the vehicle group, A_{2A}R
354 antagonist shortened the duration of propofol-induced LOC by significantly increasing *c-Fos*
355 expression in DR. These results are in agreement with the abovementioned studies indicating that
356 neurons in DR play an important role during sleep wakefulness procedures. A_{2A}R agonist prolonged
357 the duration of unconsciousness induced by propofol, but there was no statistical difference in *c-*
358 *Fos* expression compared to the vehicle group. These results are interesting and explainable. A

359 possible explanation for this might be the administration of a larger induction dose of propofol
360 (15mg/kg, IV), which severely suppressed the expression of *c-Fos* in the vehicle group (as shown
361 in Fig. 5). Although CGS prolonged the time of LOC induced by propofol, the expression of *c-Fos*
362 cannot be further reduced due to the presence of the “floor effect”.

363 NAc consists of two types of neurons, including GABAergic projection neurons and interneurons,
364 of which GABAergic projection neurons are divided into enkephalinergic and dynorphinergic
365 neurons. A_{2A}R is mainly expressed in GABAergic enkephalinergic neurons and associated with the
366 indirect efferent pathway of the basal ganglia system (Schiffmann *et al.* 2007; Ferre *et al.* 2007).
367 Therefore, the projected target region of NAc neurons does not only represent the region projected
368 by NAc A_{2A}R neurons. As Cre-LoxP technology is widely used to trace specific types of neurons,
369 projected brain regions of A_{2A}R neurons in NAc were tracked, including STR, ventral tegmental
370 area (VTA) and DR (Zhang *et al.* 2013). It is worth noting that the projection between NAc and DR
371 is not to a single direction. Muzerelle and colleagues confirmed that 5-HT neurons in the DR could
372 project to multiple brain regions, such as the hypothalamus, STR, VTA and NAc (Muzerelle *et al.*
373 2016). Based on the above-mentioned research and the results of the rsfMRI and *c-Fos* staining in
374 the current study, the connection or interaction between NAc and DR may play a key role in the
375 LOC induced by propofol and is worthy of further research.

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

381 neurons should, therefore, be identified in future. Fourth, although CGS21680 has been introduced
382 as a selective and potent adenosine A₂ receptor agonist with approximately 140-fold selectivity for
383 A₂ over A₁ receptors in the rat brain (Hutchison *et al.* 1989), its effect on the A₁ receptor does exist.
384 Moreover, A₁R activation causes profound sedation (Dunwiddie & Worth 1982) and increases the
385 duration of LOC induced by halothane anesthesia (Tanase *et al.* 2003). In this study, the effect of
386 A₁R activation induced by CGS21680 could not be completely excluded. Thus, the difference in
387 activation of A₁R and A_{2A}R induced by CGS 21680 and the duration of propofol-induced LOC
388 should be further investigated.

389

390 **5 Conclusion**

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

399

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406

407 **Conflict of interest**

408 The authors declare no competing financial interests.

409

410 **Reference**

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579

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 \pm 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, retrospliental 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 μ m. J: Statistical analysis of *c*-Fos expression in NAc (Mean \pm 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 μ m. J: Statistical analysis of *c*-Fos expression

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