Toxicological effects of propofol abuse on the dopaminergic neurons in ventral tegmental area and corpus striatum and its potential mechanisms

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ABSTRACT — This study was aimed at examining propofol-induced emotion-related behavioral disorders in mice, and exploring the possible molecular mechanisms. A total of 60 mice were divided into two groups: control and propofol group. Mice were injected with propofol (150 mg/kg, ip) at 8:00 a.m. (once a day, lasting for 30 days). During the 30 days, loss of righting reflex (LORR) and return of righting reflex (RORR) of mice were recorded every day. At the 1st (T1) and 30th (T2) day of drug discontinuance (T2), 15 mice of each group were selected to perform the open field test; then the mice underwent perfusion fixation, and the midbrain and corpus striatum were separated for immunofluorescence assay with anti-tyrosine hydroxylase (Th) and anti-dopamine transporter (DAT) antibodies. Results showed that after propofol injection, LORR and RORR increased and decreased, respectively. Long-term use of propofol resulted in decreased activities of mice (activity trajectory, line crossing, rearing time, scratching times and defecating frequency). Immunofluorescence assay showed long-term use of propofol induced decrease of Th and DAT. Collectively, our present work suggested long-term abuse of propofol induces neuropsychiatric function impairments, and the possible mechanisms are related to dopamine dysynthesis via down-regulating tyrosine hydroxylase and dopamine transporter.

Key words: Propofol, Abuse, Ventral tegmental area, Dopamine, Dopaminergic neurons

INTRODUCTION

Propofol, an intravenous ultrashort acting anesthetic, is one of the most commonly used anesthetic agents for induction and maintenance of anesthesia, procedural, and critical care sedation clinically due to its good sedative effects. Consequently, propofol is also commonly used for pediatric anesthesia and sedation (Chidambaran et al., 2015; Sommerfield et al., 2019). However, previous investigations also revealed that long-term administration of propofol may result in a series of side-effects, such as injection pain, anaphylaxis, respiration and circulation suppression, and propofol infusion syndrome, etc. (Bonnet and Scherbaum, 2012; Bolkenius et al., 2018; Stoker and Barker, 2016)

Furthermore, a growing number of studies have indicated that drug abuse of propofol may also lead to neuropsychiatric impairment, and it has been reported that propofol abuse is correlated to the propofol-induced effects of relieving stress and maintaining a sense of happiness (Bonnet and Scherbaum, 2012; Chidambaran et al., 2015). Previous investigations suggested that long-term injection of propofol may induce abnormal feelings, such as depression, anxiety, and moodiness, etc. (Chidambaran et al., 2015; Stoker and Barker, 2016) Dopaminergic neurons in the ventral tegmental area (VTA) play an important role in the development of drug addiction, anxiety and depression (Morales and Margolis, 2017; Sahinovic
et al., 2018). Thus, we speculated that the long-term injection of propofol induced depression, anxiety and moodiness might be related to the dopaminergic neurons impairment in VTA. Consequently, the present study aimed to investigate the emotion-related behavioral disorders in mice induced by long-term injection of propofol, and explore the possible molecular mechanisms.

**MATERIALS AND METHODS**

**Animals**

Male C57BL/6 mice (22-25 g) were supplied by the Dashuo Experimental Animal Center (Chengdu, China). They were housed at 21 ± 1°C under a 12-hr light/dark cycle and had free access to standard pellet diet (Purina chow) and tap water. All animal treatments were performed strictly in accordance with international ethical guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experiments were carried out with the approval of the Animal Experimentation Ethics Committee of Chengdu Medical College (Approval No. 2017090001).

**Chemicals and reagents**

Propofol injection was purchased from Corden Pharma S.P.A. (Zurich, Switzerland); anti-Tyrosine Hydroxylase (Th) antibody (catalog number: AB152) was purchased from Millipore (Boston, MA, USA); DAPI was purchased from Beyotime Biotechnology (Haimen, China); Alexa Fluor® 488 goat anti-rabbit IgG antibody was purchased from Promega Corporation (Madison, WI, USA); anti-DAT antibody was purchased from Abcam Co. (Cambridge, UK).

**Animal grouping and protocols**

A total of 60 male C57BL/6 mice were randomly divided into two groups: propofol group (P group) and control group (C group). For the propofol group, mice were injected with propofol intraperitoneally at 8:00 a.m. at the dose of 150 mg/kg (ip, once a day, lasting for 30 days), which is a commonly used dose in experimental mice (in our preliminary experiment, a 100% LORR incidence in mice was observed with propofol treatment at the dose of 150 mg/kg); for the control group, mice were received the same injection with normal saline instead of propofol for 30 days. During the 30 days, loss of righting reflex (LORR) and return of righting reflex (RORR) of the mice were recorded every day. At the 1st (T1) and 30th (T2) day after drug discontinuance, 15 mice of each group were selected to carry out the open field test. After the open field test, the mice were underwent perfusion fixation, subsequently the midbrain and corpus striatum tissues were separated and then fixed with 4% paraformaldehyde (PFA) at 4°C for the further analysis (Fig. 1).

**Loss and return of righting reflex test**

After propofol injection (150 mg/kg, ip), the loss of righting reflex (LORR) and return of righting reflex (RORR) of the mice were evaluated every day using a timer. The loss of righting reflex (LORR) was treated as the start of anesthetic sleeping state, and the return of righting reflex was treated as the consciousness recovery.

**Open field test**

The open field test was carried out using a four-compartment open field experimental video analysis system, and the experimental methods were carried out according to the previously reported method with minor modifications (Mao et al., 2009; Shahzadi et al., 2018). Mice were
placed in the center of the open field box and the activity video of the mice was recorded within 5 min, and each mouse was tested 5 times. The mood-related behavioral parameters were recorded during the mouse experiments, such as the number of line crossing times, rearing times, searching times and defecating times. After one mouse experiment was completed, the open field was cleaned and deodorized. Then, the activity video of the mice was analyzed by using the video analysis system.

**Immunofluorescence examination**

The fixed brain tissues were dehydrated with 30% saccharose, and embedded in liquid nitrogen. The embedded brain tissues were sectioned into 20 μm under -20°C. Then, the tissue sections were blocked with goat serum for 30 min, then the sections were incubated with the primary antibodies of TH (dilution 1:200) and DAT (dilution 1:200) overnight at 4°C. Subsequently, thelexa Fluor® 488 goat anti-rabbit IgG secondary antibody was applied, and finally the DAPI used to stain the cell nucleus. The immunofluorescence results were examined using a fluorescence microscope (Olympus, Tokyo, Japan).

**Western blotting assays**

Total proteins were grinded and extracted using cell lysis buffer, and the protein concentration determined by BCA protein assay reagent (Beyotime). Then, the total proteins (40 μg) were separated by SDS/PAGE, and subsequently transferred to PVDF membrane. The PVDF membrane was incubated with the primary antibodies of DAT (dilution: 1:2000), TH (dilution: 1:2000) and GAPDH (Beyotime; dilution: 1:200), and followed by incubation with secondary antibody (Beyotime). Finally, the protein bands were detected by chemiluminescence. Image J software was used to analyze the results.

**Statistical analysis**

Data were expressed as mean ± standard deviations (S.D.). Statistical comparisons were made by one-way analysis of variance (ANOVA) using SPSS software (version 18.0, USA), followed by Dunnett multiple comparison test. P value less than 0.05 was considered as significant.

**RESULTS**

**Results of LORR and RORR tests**

Figure 2 summarizes effects of long-term use of propofol on LORR and RORR. For the propofol-treated mice, following the increasing injection time, the LORR time showed an obvious increased tendency, whereas the ROSS time exhibited a significant decreased tendency.

**Results of the open field test**

Results of the open field test are shown in Fig. 3, and the results indicated that both at the 1st and 30th day after drug discontinuance, the activities of propofol-treated mice were obviously decreased, compared to the control mice. Furthermore, long-term use of propofol could decrease the line crossing (p < 0.01), rearing time (p < 0.01), scratching times (p < 0.01), defecating frequency (p < 0.01), travel distance (p < 0.01) and mobile time (p < 0.01) respectively, compared to the control mice.

**Propofol decrease the dopaminergic neurons in VTA of mice**

As can be seen in Fig. 4, dopaminergic neurons in VTA of mice were showed. Compared to the control mice, after long-term injection of propofol, the dopaminergic neurons in VTA (Fig. 4) of mice were decreased obviously both at the 1st and 30th day after drug discontinuance. There was no significant difference in counts of midbrain VTA TH positive dopaminergic neurons (p > 0.05).

Propofol decrease the Tyrosine Hydroxylase in corpus striatum tissues of mice

Effects of propofol on dopamine transporter in corpus striatum tissues of mice

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striatum tissues of mice are shown in Fig. 5. Compared to the control mice, after long-term injection of propofol, the dopaminergic neurons in corpus striatum tissues of mice were decreased obviously both at the 1st and 30th day after drug discontinuation. Upon further analysis of the fluorescence intensity value of the Th-positive area by Image J software, it was found that the fluorescence intensity value in the Th-positive area of the striatum of group P mice was significantly lower than that of group C at either T1 or T2 (p < 0.01). In addition, the results of immunofluorescence for Th were confirmed by further western blotting assay (Fig. 5).

Propofol decrease the dopamine transporter in corpus striatum tissues of mice

Effects of propofol on dopamine transporter in corpus striatum tissues of mice are shown in Fig. 6. Similarly, compared to the control mice, after long-term propofol injection, the dopaminergic transporter (DAT) in corpus striatum tissues of mice were decreased obviously both at the 1st and 30th day after drug discontinuation. Following further analysis of the fluorescence intensity value of the DAT-positive area by Image J software, it was found that the fluorescence intensity value in the DAT-positive area of the striatum of group P mice was significantly lower than that of group C at either T1 or T2 (p < 0.01). In addition, the results of immunofluorescence for DAT were confirmed by further western blotting assay (Fig. 7).
DISCUSSION

Propofol is a widely applied intravenous anesthetic agent in modern anesthetic practice, and however, previous clinical investigations also found that long-term use of propofol also results in many troublesome side-effects, in particular neuropsychiatric impairments (Bonnet and Scherbaum, 2012; Collins, 2017; Monroe et al., 2011). Similar to morphine, it has been reported that propofol injection can produce euphoria and happiness for people, which is the main reason for the long-term abuse of this anesthetic agent (Bonnet and Scherbaum, 2012). However, the detailed molecular mechanisms are still unclear.

Current research found that propofol could affect the electrophysiological activity of dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain, indicating that VTA might be the potential target of propofol (Kim et al., 2015). The VTA is a crucial area for the development of drug addiction and tolerance and regulation of emotion; additionally, the impairments of dopaminergic neurons in VTA are closely correlated to the depressive disorder, anxiety and attention-deficit, etc. (Alvarsson et al., 2016; Espana and Jones, 2013; Germann et al., 2016; Morales and Margolis, 2017;}

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Sahinovic et al., 2018). The clinical investigation by Bonnet et al. found that long-term use of propofol could result in some moodiness, anxiety, and depression, etc. (Bonnet and Scherbaum, 2012), which was similar to the phenomenon of impairments of dopaminergic neurons (Munhall et al., 2012). Consequently, we speculated that the neuropsychiatric impairments induced by long-term injection of propofol, such as depression, anxiety and moodiness, might be correlated to the dopaminergic neuron impairment in VTA. It has been reported that animal behavioral experiments are effective way for investigating the pathological changes and pathogenesis of various neuropsychiatric disorders of humans (Peng et al., 2019; Xue et al., 2014). In this study, we determined the emotion-related behavior parameters, including activity trajectory, line crossing, rearing time, scratching times, and defecat-

Fig. 5. Effects of long-term use of propofol on dopaminergic neurons in corpus striatum tissues of mice (×10). Th represents dopaminergic neuron staining in ventral striatum, Merge represents DAPI (blue) and Th (green) combine channels. (a) and (b) represent the 1st (T1) and 30th (T2) day after drug discontinuance, respectively. (c) represents comparison of Th immunofluorescence intensity of striatum in the two groups of mice. Below the dotted line is the ventral striatum. Data are expressed as mean ± SD (n = 5), **p < 0.01, vs. control mice.
ing frequency via the open field test with the experimental video analysis system. The present results showed that long-term injection of propofol resulted in a decrease in the activities of the mice, including activity trajectory, line crossing, rearing time, scratching times, and defecating frequency, indicating that long-term use of propofol might induce some emotional disorders, such as anxiety and depression. Importantly, our results also showed that the propofol-induced emotional disorders cannot be reversed by long-term drug discontinuance.

To explore the further molecular mechanisms corresponding to the side-effects of propofol, immunofluorescence assay staining with anti-tyrosine hydroxylase (Th) and anti-dopamine transporter (DAT) antibodies was carried out. Tyrosine hydroxylase (Th), a rate-limiting enzyme for catecholamine neurotransmitter synthesis...
sis in mammals, can catalyze the L-tyrosine into L-dopa, and the dopamine could be further synthesized using L-dopa via decarboxylation. DA exists in the storage particles of dopaminergic neuron and is excreted in the synaptic cleft, and then the DA would play its physiological functions (Daubner et al., 2011; Witkovsky et al., 2009; Zhu et al., 2012). After the end of a signal transduction, the DA could be reuptaken into the dopaminergic neuron by the dopamine transporter (DAT). Thus, the DAT plays a very important role in maintaining the normal concentration of DA as well as the functions of neurons (Foster and Vaughan, 2017; Ludwig et al., 2016; Nirenberg et al., 1996). After long-term injection of propofol, a significant decrease in Th expression in the VTA region of the midbrain is indicative of a disorder of dopamine transmitter synthesis. With DA synthesis disorder, DA neurotransmitters stored in dopaminergic neurons are released to the presynaptic membrane and the amount of synaptic space is significantly reduced, the total amount of DA that needs to be reuptaken into the cell is reduced, and the expression of DAT in the presynaptic membrane decreases with the decrease of the substrate DA concentration, which is consistent with our observations. Based on the results of the above experiments, it is not difficult to find that although long-term injection of propofol did not reduce the number of dopaminergic neurons in the VTA region of the midbrain, the biological function of dopaminergic neurons had significant damage. Long-term injection of propofol can cause dopamine neurotransmitter anabolic disorders, and eventually cause neuropsychological changes in mice. Th is a key enzyme in the synthesis of the neurotransmitter DA. Tyrosine hydroxylase (Th) is a key rate-limiting enzyme in the synthesis of the neurotransmitter DA (Daubner et al., 2011). Long-term use of propofol(225,736),(295,991) may cause the down-regulated expression of Th, and further lead to reduced DA synthesis.

The results of our study revealed that long-term use of propofol decreased the contents of tyrosine hydroxylase (Th) and dopamine transporter (DAT) in VTA and corpus striatum tissues of mice. The results mentioned above suggested that long-term use of propofol may result in the dysynthesis of dopamine neurotransmitter. Importantly, our results also showed that the propofol-induced decrease of Th and DAT cannot be reversed by long-term drug discontinuance.

In conclusion, our present work suggested that long-term abuse of propofol could induce neuropsychiatric function impairments, and the possible mechanisms are related to dysynthesis of dopamine via down-regulating tyrosine hydroxylase and dopamine transporter. Our
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results may be beneficial for further exploration of the toxicological mechanisms of propofol.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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