



Enhanced AMPA receptor-mediated excitatory transmission in the rodent rostromedial tegmental nucleus following lesion of the nigrostriatal pathway

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ABSTRACT

The GABAergic rostromedial tegmental nucleus (RMTg) has reciprocal connections with the dopaminergic ventral tegmental area and substantia nigra pars compacta (SNc), and is involved in inhibitory control of monoaminergic nuclei. At present, it is not clear whether unilateral 6-hydroxydopamine lesions of the SNc in rats affect AMPA receptor-mediated excitatory transmission in the RMTg. Here we found that lesions of the SNc in rats increased the firing rate of GABAergic neurons and the level of glutamate in the RMTg compared to sham-operated rats. Intra-RMTg injection of AMPA receptor agonist (S)-AMPA increased the firing rate of the GABAergic neurons in both sham-operated and the lesioned rats, while AMPA receptor antagonist NBQX decreased the firing rate of the neurons. Further, intra-RMTg injection of (S)-AMPA decreased the levels of dopamine and serotonin in the medial prefrontal cortex (mPFC) in the two groups of rats; conversely, NBQX increased the levels of dopamine and serotonin. Compared to sham-operated rats, the duration of (S)-AMPA and NBQX action on the firing rate of GABAergic neurons in the RMTg and release of dopamine and serotonin in the mPFC was prolonged in the lesioned rats. In addition, lesions of the SNc in rats increased protein expression of t-GluR1 and p-GluR1-S831 subunits compared to sham-operated rats. Therefore, these changes in the lesioned rats are associated with increased release of glutamate and up-regulated expression of GluR1 subunit-containing AMPA receptors in the RMTg, which suggest that degeneration of the nigrostriatal pathway enhances AMPA receptor-mediated excitatory transmission in the RMTg.

1. Introduction

The mesopontine rostromedial tegmental nucleus (RMTg), also known as the tail of the ventral tegmental area (tVTA), is a newly discovered structure that contains mainly GABAergic neurons (Jhou et al., 2009; Kaufling et al., 2009). It receives densely glutamatergic afferents from the lateral habenula (LHb; Jhou et al., 2009; Kaufling et al., 2009; Yetnikoff et al., 2015; Petzel et al., 2017), and projects strongly to midbrain monoaminergic nuclei, including the VTA and substantia nigra pars compacta (SNc; Jhou et al., 2009; Kaufling et al., 2010; Balcita-Pedicino et al., 2011; Bourdy et al., 2014), and the raphe nuclei (Kaufling et al., 2010; Lavezzi et al., 2012; Segó et al., 2014). Further, the stimulation of the RMTg inhibits midbrain dopaminergic neurons, while its inhibition increases their firing (Hong et al., 2011; Lecca et al., 2011, 2012; Bourdy et al., 2014), suggesting that the RMTg

may be an inhibitory control center for dopaminergic neuron activity. Based on the afferent and efferent projections of the RMTg, it is thought to be involved in the mechanisms of aversion and reward. In addition, dysfunction of the RMTg may contribute to the pathophysiology of mental disorders such as depression and addiction.

Glutamate α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors are tetrameric structures composed of combinations of subunits named GluR1–GluR4, which are distributed in the central nervous system (Keinanen et al., 1990). These receptors mediate most of the excitatory transmission and also participate in forms of synaptic plasticity thought to underlie learning and memory. The pathology of Parkinson's disease (PD) is not solely restricted to the nigrostriatal pathway and dopaminergic transmitter system, and the neural degeneration also causes abnormalities of other brain structures and transmitter systems. Further, changes in AMPA receptor expression and

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binding have been observed in parkinsonian animals and PD patients, and these studies reported increased, decreased or unchanged GluR1–GluR4 subunit expression (Porter et al., 1994; Tremblay et al., 1995; Bernard et al., 1996; Betarbet et al., 2000; Lai et al., 2003; Ba et al., 2006; Ouattara et al., 2010; VanLeeuwen et al., 2010; Kintz et al., 2013; Koutsokera et al., 2014; Xue et al., 2014) and the receptor binding sites (Wullner et al., 1993, 1994; Zavitsanou et al., 1996) in the different structures of the basal ganglia. Our recent studies have found that 6-hydroxydopamine (6-OHDA) lesions of the SNc in rats lead to an increase in the firing activity of Lhb glutamatergic neurons (Han et al., 2015; Wang et al., 2017). Despite the RMTg receives excitatory inputs from the Lhb and expresses AMPA receptors, there are no studies that have investigated changes of AMPA receptor-mediated excitatory transmission in the RMTg after unilateral lesions of the nigrostriatal pathway in rats. Therefore, the aim of the present study was to (i) examine how unilateral 6-OHDA lesions of the SNc in rats alter the firing rate and pattern of GABAergic neurons in the RMTg and their response to AMPA receptor stimulation, and the levels of extracellular GABA and glutamate in the RMTg, (ii) determine change in protein expression of GluR1 subunit, and (iii) determine whether activation and blockade of AMPA receptors in the RMTg affect release of two monoamines dopamine (DA) and serotonin (5-HT) in the medial prefrontal cortex (mPFC).

2. Materials and methods

2.1. Animals and drugs

Male Sprague-Dawley rats (280–330 g; Experimental Animal Center of Xi'an Jiaotong University, Xi'an, China) were housed in groups of four under a 12-h light-dark cycle with access to food and water *ad libitum*. All experimental procedures were approved by the Animal Care Committee of Xi'an Jiaotong University and were in accordance with NIH guidelines. All efforts were made to minimize animal suffering and the number of animals that were used.

Desipramine hydrochloride, 6-OHDA hydrochloride and apomorphine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). (*S*)- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [(*S*)-AMPA; selective AMPA receptor agonist] and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo [*f*]quinoxaline-7-sulfonamide (NBQX; potent AMPA receptor antagonist) were purchased from Tocris Bioscience (Bristol, UK). Desipramine, (*S*)-AMPA and NBQX were dissolved in saline; 6-OHDA and apomorphine were prepared in saline containing 0.02% ascorbic acid. These drugs were prepared on the day of the experiment. The doses of (*S*)-AMPA and NBQX were chosen on the results of a pilot experiment.

2.2. 6-OHDA lesion surgeries

Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.), placed on a stereotaxic frame (SN–2N; Narishige, Tokyo, Japan) and received desipramine (25 mg/kg, i.p.; 30 min before 6-OHDA injection) to protect noradrenergic neurons. 6-OHDA (12 μ g/4 μ l) was injected over 5 min into the right SNc (AP – 5.0 mm, ML 2.0 mm, DV 7.3 mm relative to bregma; Paxinos and Watson, 2004). Sham-operated rats received 4 μ l of saline containing 0.02% ascorbic acid in the same procedure. One week after surgery, the test of rotational behavior induced by apomorphine (0.05 mg/kg, s.c.) was used to assess the effect of the 6-OHDA lesion, and rats exhibiting more than 20 contralateral turns per 5 min were collected for the further study (Han et al., 2015; Wang et al., 2017). All rats used in this study turned consistently towards the side contralateral to the side of the lesion of > 35 turns per 5 min. Sham-operated rats as control did not undergo desipramine injection and apomorphine-induced rotation. In this study, all experiments were performed during the fourth week after surgery.

2.3. In vivo electrophysiology

Extracellular single-unit recording and local drug injection were performed in sham-operated and the 6-OHDA-lesioned rats, as previously described (Lecca et al., 2011; Han et al., 2015; Wang et al., 2017). Rats were anesthetized with urethane (1 g/kg, i.p.) and fixed in a stereotaxic frame (SN–2N; Narishige), and body temperature was maintained at 37 °C using a feedback-controlled heating pad. Two glass micropipettes, with one for recording (impedance, 5–10 M Ω) and one for local drug injection were used. The recording micropipette was filled with 2% pontamine sky blue in 0.5 M sodium acetate and the injection micropipette was filled with (*S*)-AMPA or NBQX. The micropipettes were lowered stereotaxically into the right RMTg (AP – 6.8–7.4 mm, ML 0.6–0.8 mm, DV 6.5–7.6 mm relative to bregma; Paxinos and Watson, 2004). Extracellular spikes were amplified (MEZ-8301; Nihon Kohden, Tokyo, Japan), bandpass-filtered using a pre-amplifier (AVB-11A; Nihon Kohden), displayed on an oscilloscope (VC-11; Nihon Kohden) and stored in a computer equipped with the CED1401 Spike 2 analysis system (Cambridge Electronic Design, England) for off-line analysis. The putative GABAergic neurons in the RMTg were identified by established electrophysiological criteria (Jhou et al., 2009; Jalabert et al., 2011; Lecca et al., 2011), including a high spontaneous firing rate (> 10 spikes/s) and a biphasic action potential of narrow duration (< 1.5 ms; measured from start to positive trough). The single-spike neuronal activity with a high signal-to-noise ratio (≥ 3) and stable firing was recorded for 5 min before the drug injection, and then (*S*)-AMPA (3 ng/40 nl) or NBQX (70 ng/40 nl) was injected into the right RMTg and the firing activity was recorded for 30 min. According to a previous report, this volume did not diffuse into the adjacent structures of the RMTg (Celada et al., 2001). In this study, only one neuron was observed per rat. At the end of each experiment, the recording site was marked by the ejection of pontamine sky blue through the recording micropipette (– 20 μ A, 10 min).

2.4. In vivo microdialysis

Microdialysis procedures were performed, as previously described (Wang et al., 2017). Under chloral hydrate anesthesia (400 mg/kg, i.p.), rats were stereotaxically implanted with guide cannula (Eicom, Kyoto, Japan) in the right RMTg (AP – 7.1 mm, ML 0.7 mm, 6.5 mm relative to bregma; Paxinos and Watson, 2004) for microdialysis. In experiments involving the local drug injection, rats were implanted with two guide cannulae, one in the right RMTg (AP – 7.1 mm, ML 0.7 mm, 6.3 mm relative to bregma; Paxinos and Watson, 2004) for drug injection and the other in the right mPFC (AP 3.3 mm, ML 0.7 mm, DV 2.2 mm relative to bregma; Paxinos and Watson, 2004) for microdialysis. The guide cannulae were secured to the skull with dental cement. After 24–48 h recovery period, microdialysis experiments were performed in unanesthetized and freely moving rats. Microdialysis probes (membrane length 2 mm; Eicom) were inserted through the guide cannula and perfused at a constant flow of 1 μ l/min with Ringer buffer (147 mM NaCl, 4 mM KCl, 2.3 mM CaCl₂) for at least 2 h before sample collection.

The contents of extracellular GABA and glutamate in the RMTg and DA and 5-HT in the mPFC were measured by high-performance liquid chromatography with electrochemical detection, as described by Wang et al. (2017). For the measurement of extracellular GABA and glutamate in the RMTg, successive 60 min dialysate samples were collected. After precolumn derivatization with *o*-phthalaldehyde and sodium sulphite for 10 min (Rowley et al., 1995), the concentrations of GABA and glutamate in 20 μ l derivatized mixture were determined. To determine whether activation and blockade of AMPA receptors in the RMTg affect release of DA and 5-HT in the mPFC, the dialysates were collected every 10 min for 60 min after intra-RMTg injection of (*S*)-AMPA (0.0375 μ g/0.3 μ l) or NBQX (0.5 μ g/0.3 μ l), and analyzed simultaneously for DA and 5-HT. Successive three 10-min dialysate samples served to obtain basal values before the local injection of the drugs. In addition, tissue

content of DA in the right striatum of rats used in Western blotting was measured, as previously described (Han et al., 2015).

2.5. Western blotting

Western blotting was used to analyze expression levels of total GluR1 subunit (t-GluR1) and phosphorylated GluR1 subunit at serine-831 site (p-GluR1-S831) in the RMTg of the two groups of rats. Briefly, rats were sacrificed and their brains were removed rapidly, and then the region of the brain that contains the RMTg (AP, -6.8 mm to -7.4 mm from bregma) was sectioned and collected the right RMTg using a punch according to the anatomic marks (Paxinos and Watson, 2004) and stored frozen at -80 °C until processed. On the day of the experiment, tissue samples were homogenized on ice in radio-immunoprecipitation assay buffer supplemented with protease inhibitors. The homogenates were centrifuged at 15,000 rpm for 15 min at 4 °C, and supernatants were collected for further analysis. The protein concentration was determined by spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific, Wilmington, DE, USA). Samples were denatured by boiling before being loaded (80 μ g) on the 8% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) and non-specific protein binding was blocked with 5% non-fat milk for 1 h at room temperature, followed by an overnight incubation at 4 °C with the following primary antibodies: anti-t-GluR1 (rabbit monoclonal, 1:1000; Abcam, Cambridge, MA, USA), anti-p-GluR1-S831 (rabbit monoclonal, 1:2000; Abcam) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; mouse monoclonal, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The next day, the membranes were washed several times and incubated with goat anti-rabbit IgG (1:2000; SAB, College Park, MD, USA) or anti-mouse (1:2000; SAB) IgG antibody conjugated with horse radish peroxidase for 2 h at room temperature. The corresponding bands were detected with the enhanced chemiluminescence (Amersham Life Science; Piscataway, NJ, USA) and visualized by exposing the membrane to autoradiography films (Fuji; Tokyo, Japan). Images of the Western blotting protein bands were collected and analyzed using NIH ImageJ 1.49 analysis software. Pixel intensities of the bands obtained in each experiment were normalized using GAPDH signals, and then calculated as a percentage of the mean of three control bands in the same membrane.

2.6. Histology and immunohistochemistry

After the electrophysiological and microdialysis experiments, rats were anesthetized by an overdose of urethane and transcardially perfused with saline, followed by 4% paraformaldehyde. Brains were removed and placed in 4% paraformaldehyde for 4 h, and then were cytoprotected in 30% sucrose solution until sunken. Coronal brain sections were cut with a cryostat (40 μ m). The sections were stained with cresyl violet to identify anatomical placement of the recording site, cannula and probe. To determine the extent of dopaminergic neurons degeneration in the SNc and VTA of sham-operated and the 6-OHDA-lesioned rats, the sections were examined for immunohistochemical staining of tyrosine hydroxylase (TH), as previously described (Wang et al., 2009).

2.7. Data treatment

In this study, electrophysiological and microdialysis data were only analyzed from rats with verified anatomical placement of the recording site, cannula and probe, and almost total loss of TH immunoreactive (TH-ir) neurons in the right SNc. In addition, only rats with reduced striatal DA by $>95\%$ were considered for the analysis of Western blotting data. For electrophysiological data, basal firing activity of GABAergic neurons in the RMTg was recorded for 5 min before injection of the drugs, and the following parameters were calculated: (i) the

firing rate; (ii) coefficient of variation (COV; the ratio between standard deviation of the interspike interval and mean interspike interval, reflecting the degree of regularity of neuronal firing; Fedrowitz et al., 2003). After intra-RMTg injection of (S)-AMPA or NBQX, changes in the firing rate of the GABAergic neurons were analyzed per 5 min epoch up to 30 min. The COV of the neurons was compared in a period of 5 min before and after injection of the drugs. For microdialysis data of DA and 5-HT, the average of three successive dialysates before injection of the drugs was defined as 100% of basal transmitter release. For immunohistochemical data, the count of dopaminergic neuron bodies in the SNc and VTA was performed on representative three sections per rat, as previously described (Hou et al., 2012).

2.8. Statistics

Statistical analysis was performed using the SigmaStat (Systat, San Jose, CA, USA). Student's *t*-test, Mann-Whitney *U* test, Friedman repeated measures analysis of variance on ranks followed by Dunn's multiple tests or two-way ANOVA with repeated measures followed by Bonferroni's test was used, when appropriate. All data were reported as mean \pm SEM. A significance level of $P < 0.05$ was used for all analyses.

3. Results

3.1. TH-ir neuron counting in the SNc and VTA and tissue content of DA in the striatum after lesioning of the SNc

Unilateral injections of 6-OHDA into the SNc in rats significantly decreased the number of TH-ir neurons in both SNc and VTA compared to sham-operated rats (SNc: sham, $3.41 \pm 0.75\%$; lesioned, $95.56 \pm 0.25\%$; VTA: sham, $4.36 \pm 0.75\%$; lesioned, $48.39 \pm 3.04\%$; both $P < 0.001$, unpaired Student's *t*-test, $n = 15$ rats/group; Fig. 1A–C). The unilateral injections also significantly decreased DA level in the ipsilateral striatum compared to sham-operated rats (sham: 8010 ± 420 ng/g wet tissue; lesioned: 185 ± 25 ng/g wet tissue; $P < 0.001$, unpaired Student's *t*-test, $n = 15$ rats/group; Fig. 1D).

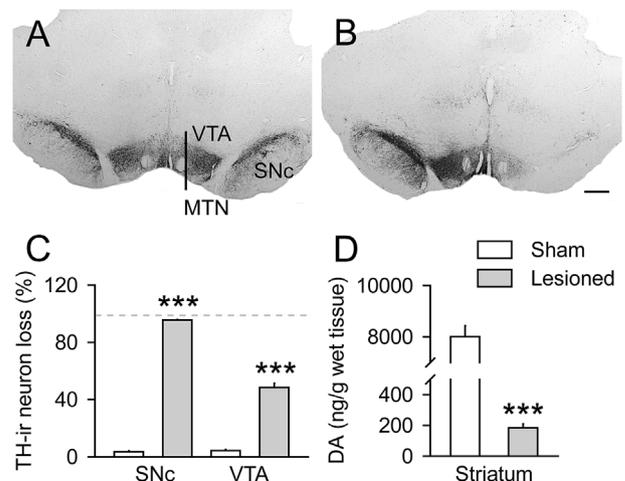


Fig. 1. TH immunostaining and DA tissue content. **A and B.** Photomicrographs showing TH immunostaining of the SNc and VTA in sham-operated (A) and the 6-OHDA-lesioned (B) rats. **C.** Unilateral Injection of 6-OHDA into the SNc in rats caused near complete and partial loss of TH-ir neurons in the SNc and VTA compared to sham-operated rats ($n = 15$ rats/group). **D.** Unilateral injection of 6-OHDA also decreased DA level in the ipsilateral striatum compared to sham-operated rats ($n = 15$ rats/group). *** $P < 0.001$ vs. sham-operated rats; unpaired Student's *t*-test. Data are reported as means \pm SEM. MTN = medial terminal nucleus of the accessory optic tract. Scale bars: A and B = 500 μ m.

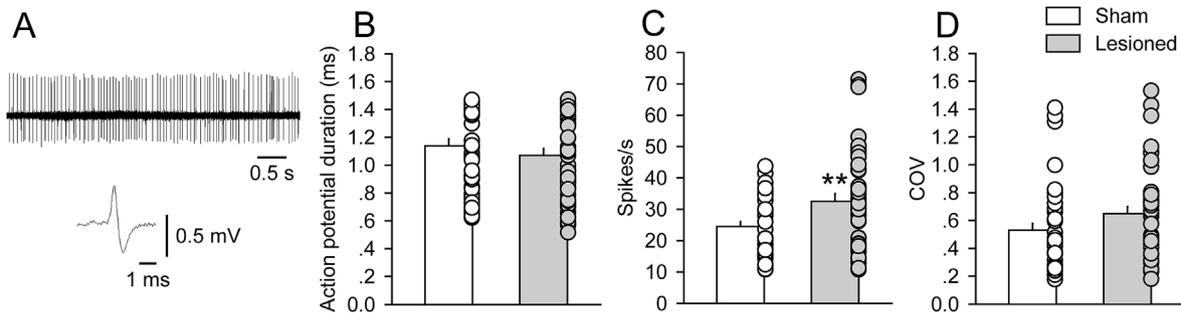


Fig. 2. The firing activity of putative GABAergic neurons in the RMTg. **A.** Extracellular recording of the representative neuron showing the spontaneous firing activity and action potential waveform in sham-operated rat. **B–D.** Histograms showing comparison of the mean action potential duration (B), mean firing rate (C) and mean COV (D) of the neurons in sham-operated and 6-OHDA-lesioned rats, and unilateral lesions of the SNc in rats ($n = 41$ neurons) increased the mean firing rate of the neurons compared to sham-operated rats (C; $n = 39$ neurons). $**P < 0.01$ vs. sham-operated rats; unpaired Student's *t*-test. Data are reported as means \pm SEM.

3.2. The firing activity of GABAergic neurons in the RMTg and their responses to AMPA receptor stimulation after lesioning of the SNc

A total of 39 RMTg neurons were recorded in sham-operated rats and 41 neurons in the 6-OHDA-lesioned rats. These neurons displayed the electrophysiological characteristics of the putative GABAergic neurons corresponding to the criteria presented in previous reports (Jhou et al., 2009; Jalabert et al., 2011; Lecca et al., 2011), and mean action potential duration of the neurons between the two groups of rats was no significant (sham: 1.14 ± 0.05 ms; lesioned: 1.07 ± 0.05 ms). The results concerning the action potential duration, firing rate and COV of the GABAergic neurons are shown in Fig. 2A–D.

In sham-operated rats, the firing rate of the GABAergic neurons ranged from 10.1 to 43.13 spikes/s with a mean of 24.49 ± 1.55 ($n = 39$ neurons; Fig. 2C), and the mean COV of these neurons was 0.53 ± 0.05 (Fig. 2D). In the 6-OHDA-lesioned rats, the firing rate of the GABAergic neurons varied from 10.1 to 70.91 spikes/s, and the mean firing rate significantly increased by 32.45 ± 2.47 spikes/s compared with that of sham-operated rats ($P < 0.01$, unpaired Student's *t*-test, $n = 41$ neurons; Fig. 2C). The mean COV of these neurons was 0.65 ± 0.06 , and was not significantly different from that of sham-operated rats (Fig. 2D). These results suggest that unilateral 6-OHDA lesions of the SNc in rats lead to an increase in the firing rate of GABAergic neurons in the RMTg.

We examined changes in the firing activity of the GABAergic neurons after intra-RMTg injection of (S)-AMPA and NBQX (Fig. 3A–C). In sham-operated rats, intra-RMTg injection of (S)-AMPA (3 ng/40 nl) significantly increased the firing rate of the GABAergic neurons (maximal effect: 164% of the baseline; $P < 0.001$, Friedman repeated measures analysis of variance on ranks, $n = 18$ neurons; Fig. 3D and F), and the excitatory effect occurred 5 min after the injection and lasted 15 min ($P < 0.05$, Dunn's test; Fig. 3F). In the 6-OHDA-lesioned rats, (S)-AMPA, at the same dose, also significantly increased the firing rate of the GABAergic neurons (maximal effect: 156% of the baseline; $P < 0.001$, Friedman repeated measures analysis of variance on ranks, $n = 21$ neurons; Fig. 3E and F), and the excitatory occurred 5 min after the injection; however, the effect lasted 25 min ($P < 0.05$, Dunn's test; Fig. 3F), which was longer than that of sham-operated rats. In contrast to the excitatory effects of (S)-AMPA, intra-RMTg injection of NBQX (70 ng/40 nl) significantly decreased the firing rate of the GABAergic neurons in sham-operated rats (maximal effect: 71% of the baseline; $P < 0.001$, Friedman repeated measures analysis of variance on ranks, $n = 19$ neurons; Fig. 3G and I), and the inhibitory effect occurred 5 min after the injection and lasted 15 min ($P < 0.05$, Dunn's test; Fig. 3I). NBQX, at the same dose, also significantly decreased the firing rate of the GABAergic neurons in the 6-OHDA-lesioned rats (maximal effect: 58% of the baseline; $P < 0.001$; Friedman repeated measures analysis of variance on ranks, $n = 20$ neurons; Fig. 3H and I). The inhibitory effect occurred 5 min after the injection; however, the effect lasted

25 min ($P < 0.05$, Dunn's test; Fig. 3I). Compared to sham-operated rats, the duration of the effect produced by NBQX was prolonged in the lesioned rats. As regards the COV, neither (S)-AMPA nor NBQX induced a change in the two groups of rats (Fig. 3J–M). These results suggest that unilateral 6-OHDA lesions of the SNc in rats increase the response of GABAergic neurons in the RMTg to AMPA receptor stimulation.

3.3. The levels of extracellular GABA and glutamate in the RMTg and monoamines in the mPFC after lesioning of the SNc and activation and blockade of RMTg AMPA receptors

The firing activity of the GABAergic neurons is affected by extracellular GABA and glutamate in the RMTg. Therefore, we measured the levels of extracellular GABA and glutamate in the two groups of rats (Fig. 4A). Unilateral 6-OHDA lesions of the SNc in rats did not significantly alter the level of GABA in the RMTg compared to sham-operated rats (sham: 2.23 ± 0.83 μ M; lesioned: 2.18 ± 0.93 μ M; Fig. 4B); however, the lesion significantly increased the level of glutamate (sham: 21.27 ± 8.12 μ M; lesioned: 78.33 ± 17.61 μ M; $P < 0.05$; unpaired Student's *t*-test, $n = 6$ rats/group; Fig. 4C). Accordingly, the GABA/glutamate ratio in the lesioned rats was significantly decreased compared to sham-operated rats ($P < 0.05$, unpaired Student's *t*-test; Fig. 4D). These results suggest that unilateral 6-OHDA lesions of the SNc in rats increase glutamate release and alter the balance of GABA and glutamate release in the RMTg.

Evidence indicates that the RMTg sends GABAergic projections to dopaminergic VTA and SNc, and serotonergic raphe nuclei (Jhou et al., 2009; Kauffling et al., 2010; Balcita-Pedicino et al., 2011; Lavezzi et al., 2012; Bourdy et al., 2014; Segó et al., 2014). To determine whether the RMTg affect the activity of monoaminergic transmitter systems, we observed changes of DA and 5-HT release in the mPFC after intra-RMTg injection of (S)-AMPA and NBQX (Fig. 5A–C). Rats with unilateral lesions of the SNc showed a significant decrease in the level of extracellular DA in the mPFC compared to sham-operated rats (sham: 14.22 ± 1.19 nM; lesioned: 8.79 ± 0.59 nM; $P < 0.001$, unpaired Student's *t*-test, $n = 12$ rats/group; Fig. 5D); however, there was no significant difference in the level of extracellular 5-HT in the mPFC between the two groups of rats (sham: 17.86 ± 0.89 nM; lesioned: 15.81 ± 0.95 nM; Fig. 5E), suggesting that unilateral 6-OHDA lesions of the SNc in rats decrease DA release in the mPFC.

Intra-RMTg injection of (S)-AMPA (0.0375 μ g/0.3 μ l) decreased the level of extracellular DA in the mPFC compared to the baseline in sham-operated (maximal effect: 64% of the baseline; Fig. 5F) and the 6-OHDA-lesioned rats (maximal effect: 64% of the baseline; $n = 6$ rats/group; Fig. 5F), respectively. A two-way ANOVA with repeated measures showed a significant effect of time ($F_{6,30} = 5.883$, $P < 0.001$), but no effect of group or time \times group interaction. In sham-operated rats, significant decrease was observed at 10 min after the injection ($P < 0.01$, Bonferroni's test; Fig. 5F). In the lesioned rats, significant

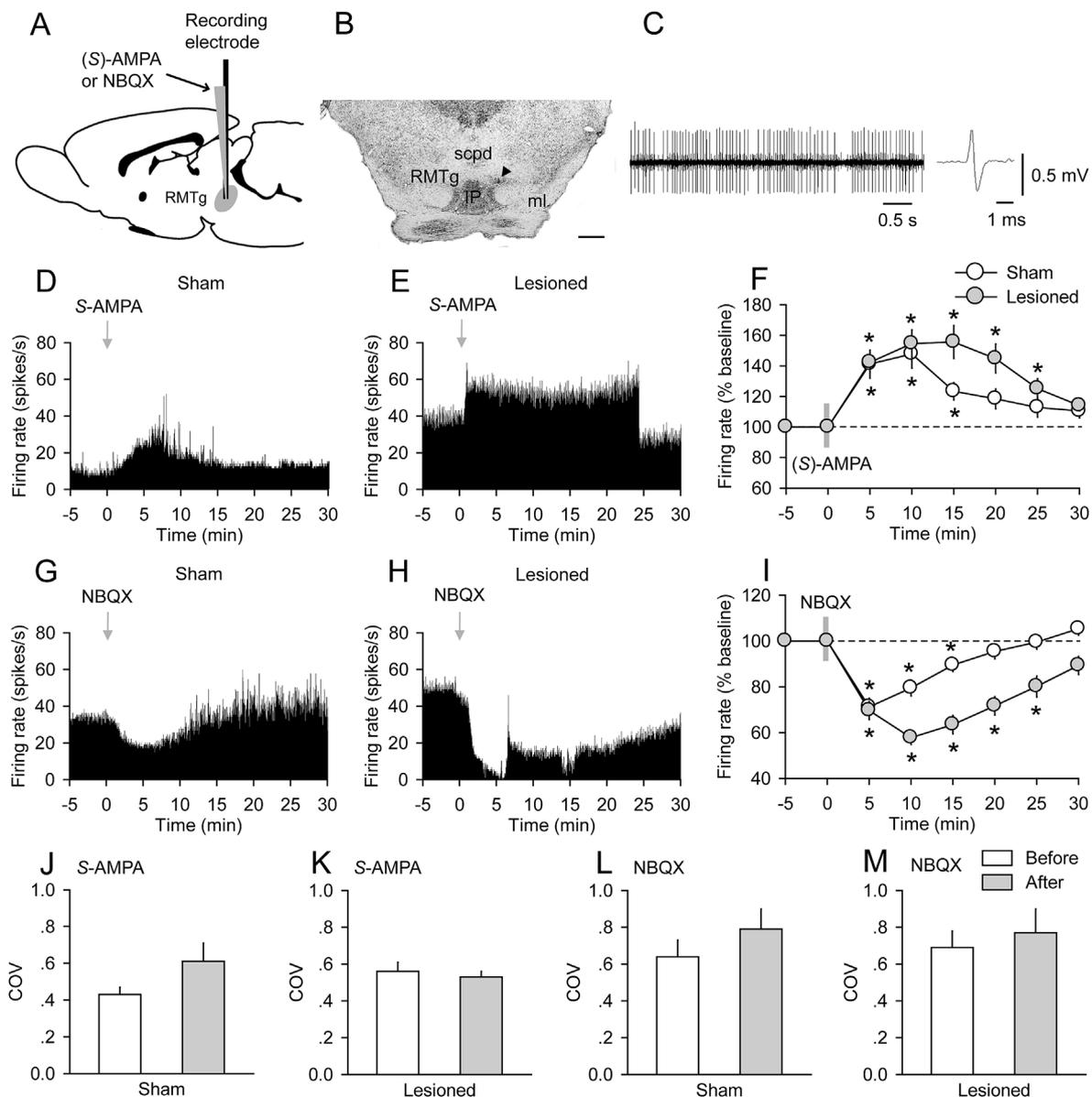


Fig. 3. Changes in the firing activity of RMTg GABAergic neurons after intra-RMTg injection of AMPA receptor agonist (S)-AMPA and antagonist NBQX. **A.** The experimental protocol. **B.** Photomicrograph of cresyl violet staining showing the recording site in the RMTg marked with iontophoretically injected pontamine sky blue in sham-operated rat (arrowhead). **C.** Extracellular recording of representative RMTg neuron showing the spontaneous firing activity and action potential waveform in rat with unilateral 6-OHDA lesion of the SNC. **D–I.** The representative firing rate histograms showing intra-RMTg injection of (S)-AMPA (3 ng/40 nl) increased the firing rate of the neurons in both sham-operated and the lesioned rats (D, E), while NBQX (70 ng/40 nl) decreased the firing rate of the neurons (G, H); although (S)-AMPA (F; sham: $n = 18$ neurons; lesioned: $n = 21$ neurons) and NBQX (I; sham: $n = 19$ neurons; lesioned: $n = 20$ neurons) induced excitatory and inhibitory effects in the two groups of rats, respectively; the duration of significant effects in the lesioned rats was shorter than those in sham-operated rats. **J–M.** Intra-RMTg injection of (S)-AMPA and NBQX did not alter the mean COV of the neurons compared to before injection in the two groups of rats. * $P < 0.05$ vs. baseline firing rate; Friedman repeated measures analysis of variance on ranks followed by Dunn's multiple tests. Data are reported as means \pm SEM. IP, interpeduncular nucleus; ml, medial lemniscus; scp, superior cerebellar peduncle, descending limb. Scale bar: B = 500 μ m.

decreases were observed at 10 ($P < 0.05$) and 20 min ($P < 0.01$, Bonferroni's test; Fig. 5F). (S)-AMPA also decreased the level of extracellular 5-HT in the mPFC compared to the baseline in sham-operated (maximal effect: 67% of the baseline; Fig. 5G) and the 6-OHDA-lesioned rats (maximal effect: 57% of the baseline; $n = 6$ rats/group; Fig. 5G), respectively. A two-way ANOVA with repeated measures showed a significant effect of time ($F_{6,30} = 15.865$, $P < 0.001$), but no effect of group or time \times group interaction. In sham-operated rats, significant decreases were observed at 10 ($P < 0.001$) and 20 min ($P < 0.05$, Bonferroni's test; Fig. 5G) after the injection. In the lesioned rats, significant decreases were observed at 10, 20 and 30 min (all $P < 0.001$, Bonferroni's test; Fig. 5G). Conversely, intra-RMTg injection of NBQX

(0.5 μ g/0.3 μ l) increased the level of extracellular DA in the mPFC compared to the baseline in sham-operated (maximal effect: 152% of the baseline; Fig. 5H) and the 6-OHDA-lesioned rats (maximal effect: 186% of the baseline; $n = 6$ rats/group; Fig. 5H), respectively. A two-way ANOVA with repeated measures showed a significant effect of time ($F_{6,30} = 25.827$, $P < 0.001$), but no effect of group or time \times group interaction. In sham-operated rats, significant increases were observed at 10 ($P < 0.001$) and 20 min ($P < 0.01$, Bonferroni's test; Fig. 5H) after the injection. In the lesioned rats, significant increases were observed at 10 ($P < 0.001$), 20 ($P < 0.001$), 30 ($P < 0.001$), 40 ($P < 0.001$) and 50 min ($P < 0.05$, Bonferroni's test; Fig. 5H). NBQX also increased the level of extracellular 5-HT in the mPFC compared to

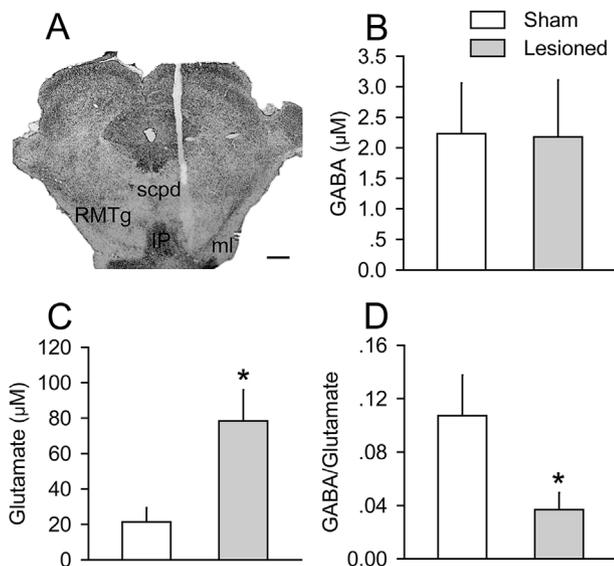


Fig. 4. The levels of extracellular GABA and glutamate in the RMTg. **A.** Photomicrograph of cresyl violet staining showing the site of microdialysis probe in the RMTg of sham-operated rat. **B and C.** Unilateral 6-OHDA lesions of the SNc in rats did not alter the level of extracellular GABA in the ipsilateral RMTg compared to sham-operated rats (B), whereas the lesion was increased the level of extracellular glutamate (C; $n = 6$ rats/group). **D.** The lesion decreased the GABA/glutamate ratio. * $P < 0.05$ vs. sham-operated rats; unpaired Student's *t*-test. Data are reported as means \pm SEM. IP, interpeduncular nucleus; ml, medial lemniscus; scpd, superior cerebellar peduncle, descending limb. Scale bar: A = 500 µm.

the baseline in sham-operated (maximal effect: 186% of the baseline; Fig. 5I) and the 6-OHDA-lesioned rats (maximal effect: 251% of the baseline; $n = 6$ rats/group; Fig. 5G), respectively. A two-way ANOVA with repeated measures showed a significant effect of time ($F_{6,30} = 49.882$, $P < 0.001$) and time \times group interaction ($F_{6,30} = 3.853$, $P < 0.01$), but no effect of group. In sham-operated rats, significant increases were observed at 10, 20 and 30 min (all

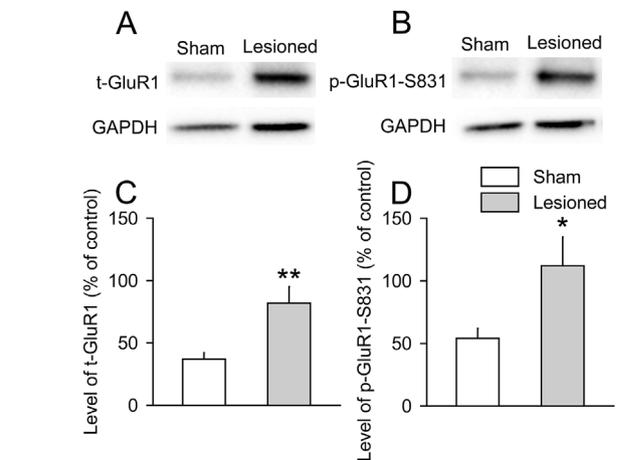


Fig. 6. Protein expression of t-GluR1 and p-GluR1-S831 subunits in the RMTg. **A and B.** Representative bands for t-GluR1 (A) and p-GluR1-S831 subunits (B) in the RMTg of sham-operated and the 6-OHDA-lesioned rats. **C and D.** Unilaterally lesioning SNc in rats increased protein level of t-GluR1 subunit in the ipsilateral RMTg compared to sham-operated rats (C; $n = 11$ rats/group), and the lesion also increased protein level of p-GluR1-S831 subunit (D; $n = 11$ rats/group). * $P < 0.05$, ** $P < 0.01$ vs. sham-operated rats; unpaired Student's *t*-test. Data are reported as means \pm SEM.

$P < 0.001$, Bonferroni's test; Fig. 5I) after the injection. In the lesioned rats, significant increases were observed at 10, 20, 30 and 40 min (all $P < 0.001$, Bonferroni's test; Fig. 5I). Further, the duration of significant effects produced by (S)-AMPA and NBQX on DA and 5-HT release in the 6-OHDA-lesioned rats was longer than that of sham-operated rats (Fig. 5F–I). These results suggest that activation and blockade of AMPA receptors in the RMTg regulate DA and 5-HT release in the mPFC of sham-operated and the 6-OHDA-lesioned rats.

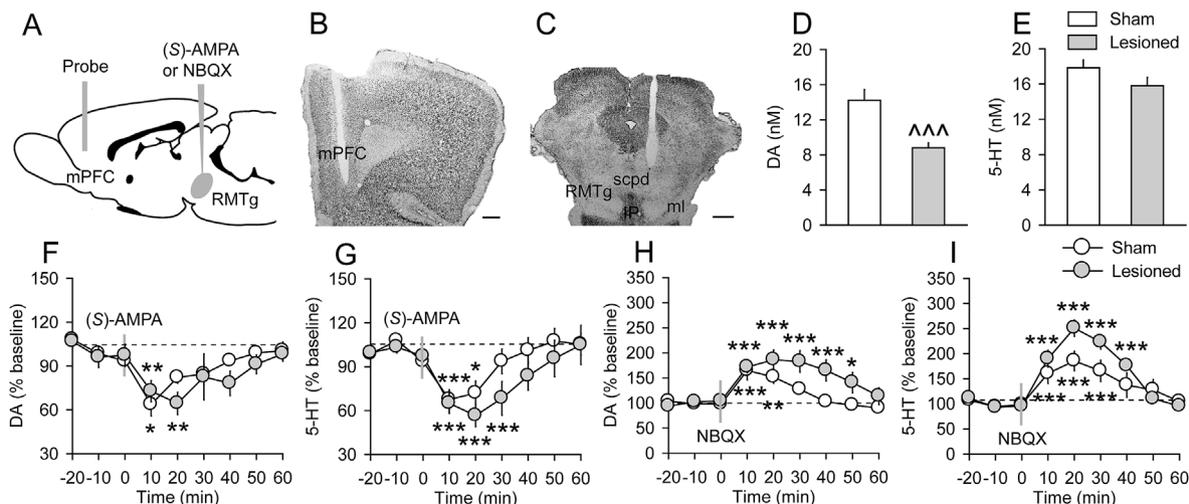


Fig. 5. Changes in the levels of extracellular DA and 5-HT in the mPFC after 6-OHDA lesion and intra-RMTg injection of AMPA receptor agonist (S)-AMPA and antagonist NBQX. **A.** The experimental protocol. **B and C.** Photomicrographs of cresyl violet staining showing the site of microdialysis probe in the mPFC (B) and the tip of the injection track aimed at the RMTg (C) of sham-operated rat. **D and E.** Unilateral 6-OHDA lesions of the SNc in rats decreased the level of DA (D) in the ipsilateral mPFC compared to sham-operated rats, whereas the lesion did not alter the level of 5-HT (E; $n = 12$ rats/group). **F–I.** Intra-RMTg injection of (S)-AMPA (0.0375 µg/0.3 µl) decreased the levels of DA and 5-HT in the mPFC of both sham-operated and the lesioned rats (F and G; $n = 6$ rats/group), while NBQX (0.5 µg/0.3 µl) increased the levels of DA and 5-HT (H and I; $n = 6$ rats/group); although (S)-AMPA and NBQX decreased or increased DA and 5-HT release in the two groups of rats, the duration of significant changes in the lesion rats was longer than that of sham-operated rats. *** $P < 0.001$ vs. sham-operated rats, unpaired Student's *t*-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. baseline, two-way ANOVA with repeated measures followed by Bonferroni's test. Data are reported as means \pm SEM. IP, interpeduncular nucleus; ml, medial lemniscus; scpd, superior cerebellar peduncle, descending limb. Scale bars: B and C = 500 µm.

3.4. Protein expression of GluR1 subunit in the RMTg after lesioning of the SNc

Because GABAergic neurons in the RMTg showed increased firing rate and enhanced responses to AMPA receptor agonist and antagonist in the 6-OHDA-lesioned rats, we used Western blotting to examine expression of GluR1 subunit in the RMTg. Compared to sham-operated rats, the results showed that unilateral 6-OHDA lesions of the SNc in rats significantly increased expression of t-GluR1 subunit (122% of control; $P < 0.01$; $n = 11$ rats/group; Fig. 6A and C) and p-GluR1-S831 subunit (107% of control; $P < 0.05$; unpaired Student's *t*-test; $n = 11$ rats/group; Fig. 6B and D) in the RMTg, suggesting that unilateral 6-OHDA lesions of the SNc in rats increase expression of t-GluR1 and p-GluR1-S831 subunits in the RMTg.

4. Discussion

The 6-OHDA rat model is commonly used in the preclinical study of PD. In order to make more selective lesions of the nigrostriatal pathway, the SNc is targeted as the site of neurotoxin 6-OHDA injection. In the present study, unilateral injection of 6-OHDA into the SNc in rats led to a near complete and partial loss of TH-ir neurons in the SNc and VTA, respectively. Therefore, the SNc lesion model partially mimics the human disease, and is similar to advanced PD (Deumens et al., 2002).

The main results of the present results show that: (i) unilateral lesions of the SNc in rats increased the firing rate of GABAergic neurons in the RMTg, and intra-RMTg injection of AMPA receptor agonist (*S*)-AMPA increased the firing rate of the neurons, while AMPA receptor antagonist NBQX decreased the firing rate of the neurons in the two groups of rats; however, the duration of the excitatory and inhibitory effects produced by (*S*)-AMPA and NBQX in the lesioned rats was markedly longer than that of sham-operated rats; (ii) the lesion increased the level of extracellular glutamate without altering the level of GABA in the RMTg, and intra-RMTg injection of (*S*)-AMPA or NBQX decreased or increased the levels of extracellular DA and 5-HT in the mPFC in the two groups of rats, and the duration of significant changes of DA and 5-HT levels in the lesioned rats was prolonged compared to sham-operated rats; and (iii) the SNc lesions in rats increased the protein levels of t-GluR1 and p-GluR1 subunits in the RMTg.

The RMTg is a recently discovered structure located in the mesopontine tegmentum, which is mostly composed of GABAergic neurons (~70%–92%; Jhou et al., 2009; Kaufling et al., 2009). The RMTg receives dopaminergic afferents from the VTA and SNc (Jhou et al., 2009). At present, there are no studies that have investigated the firing activity of RMTg GABAergic neurons in parkinsonian rats. Our results showed that unilateral 6-OHDA lesions of the SNc in rats significantly increased the firing rate of the GABAergic neurons without affecting the regularity of the neuronal firing compared to sham-operated rats. Regarding the firing activity of GABAergic neurons in the RMTg, the intrinsic cellular properties and regulation by excitatory and inhibitory inputs from other brain regions may play an important role in the firing activity of the neurons. In the present study, *in vivo* microdialysis results found that unilaterally lesioning SNc in rats significantly increased the level of extracellular glutamate in the RMTg compared to sham-operated rats, whereas the level of extracellular GABA was not altered, which provide a direct evidence for increased firing rate of RMTg GABAergic neurons in the lesioned rats. Although RMTg afferents are widely dispersed, the most studied input to the RMTg remains glutamatergic LHB (Jhou et al., 2009; Kaufling et al., 2009; Yetnikoff et al., 2015; Petzel et al., 2017). Further, recent studies have shown that unilateral 6-OHDA lesions of the SNc in rats lead to an increase in the firing activity of glutamatergic neurons in the LHB (Han et al., 2015; Wang et al., 2017). Therefore, *in vivo* microdialysis results are supported by these previous reports showing that afferents of the RMTg mostly arise from glutamatergic LHB. Based on the present results and

previous reports, we consider that the depletion of DA in rats increases glutamatergic outputs of the LHB, which subsequently increase glutamate release in the RMTg, and then increased level of glutamate induces excitation of the GABAergic neurons. In addition, decreased GABA/glutamate ratio suggests that the balance of GABA and glutamate release in the RMTg is altered in the 6-OHDA-lesioned rats.

The glutamate mediates its fast excitatory transmission through AMPA receptors. Further, a study has found that the excitatory postsynaptic currents evoked in the RMTg are mediated by AMPA receptors (Lecca et al., 2011). The present results showed that intra-RMTg injection of (*S*)-AMPA significantly increased the firing rate of the GABAergic neurons, while NBQX significantly decreased the firing rate of the GABAergic neurons in both sham-operated and the 6-OHDA-lesioned rats. In addition, neither (*S*)-AMPA nor NBQX altered the regularity of the firing activity of the GABAergic neurons. However, the duration of significant changes in the firing rate induced by (*S*)-AMPA and NBQX in the lesioned rats was markedly longer than that of sham-operated rats. In addition to increased release of glutamate, increased firing rate of RMTg GABAergic neurons and the prolonged duration of (*S*)-AMPA and NBQX action on the firing rate of the neurons also reflect the abnormal expression of AMPA receptors in the lesioned rats.

The GluR1 subunit is widely distributed in the brain, and shows phosphorylation at two distinct serine sites (831 and 845) in the C-terminus (Roche et al., 1996). Phosphorylation of either of these two sites enhances AMPA receptor ion channel function by increasing the channel open probability (Roche et al., 1996; Barria et al., 1997). In addition, GluR1 subunit serine 831 is phosphorylated by Ca^{2+} -activated calmodulin kinase II, which is crucial for synaptic plasticity (Roche et al., 1996; Barria et al., 1997). In rodent models of parkinsonism, the results of the GluR1 and p-GluR1-S831 subunits are less consistent. These studies reported decreased or unchanged expression of the GluR1 and p-GluR1-S831 subunits in the entopeduncular nucleus and striatum after degeneration of the nigrostriatal pathway (Bernard et al., 1996; Lai et al., 2003; Ba et al., 2006; Kintz et al., 2013; Xue et al., 2014). Therefore, we further examined change in protein expression of GluR1 subunit in the RMTg. Compared to sham-operated rats, the Western blotting analysis showed a significant increase in the levels of both t-GluR1 and p-GluR1-S831 subunits in the RMTg of the lesioned rats, suggesting that the depletion of DA in rats up-regulates the expression of GluR1 subunit-containing AMPA receptors in the RMTg. These findings further support the electrophysiological results showing that lesions of the SNc in rats increased firing rate of GABAergic neurons in the RMTg compared to sham-operated rats. Up-regulation of GluR1 subunit-containing AMPA receptors in the lesioned rats also confirms the results of RMTg GABAergic neurons to AMPA receptor stimulation showing that the duration of (*S*)-AMPA and NBQX action on the firing rate of the neurons was prolonged compared to sham-operated rats.

In the present study, up-regulated expression of GluR1 subunits in the RMTg of the lesioned rats enhanced the response of the GABAergic neurons to GluR1 subunit-containing AMPA receptor stimulation, which influences release of GABA in output targets of the RMTg. Compared to afferents of the RMTg, GABAergic inhibitory efferents of the RMTg are more restricted and primarily project to the dopaminergic VTA and SNc (Jhou et al., 2009; Kaufling et al., 2010; Balcita-Pedicino et al., 2011; Bourdy et al., 2014). In addition, the RMTg also innervates the serotonergic raphe nuclei including the dorsal and median raphe nucleus (Kaufling et al., 2010; Lavezzi et al., 2012; Sego et al., 2014), and projections to the dorsal raphe nucleus are focally directed to a distinctive subdivision poor in 5-HT and enriched in presumptive glutamatergic neurons (Sego et al., 2014). These studies suggest that the RMTg is involved in the regulation of the two transmitter systems. The mPFC is one of the brain regions involved in a large number of higher brain functions, and has extensive reciprocal connections with the VTA (Oades and Halliday, 1987; Sesack and Pickel, 1992) and raphe nuclei (Lidov et al., 1980; Sesack et al., 1989). Therefore, we observed

changes of DA and 5-HT release in the mPFC after intra-RMTg injection of (S)-AMPA and NBQX. The results showed that unilaterally lesioning SNc in rats significantly decreased in the level of extracellular DA in the mPFC compared to sham-operated rats, and the level of extracellular 5-HT in the mPFC was not altered, which is in agreement with a recent report (Wang et al., 2017). These results suggest that the 6-OHDA lesions of the SNc in rats decrease DA release in the mPFC. Further, intra-RMTg injection of (S)-AMPA significantly decreased the levels of extracellular DA and 5-HT in the mPFC in both sham-operated and the lesioned rats, while NBQX significantly increased the levels of DA and 5-HT, suggesting that GABAergic outputs of the RMTg inhibit the activity of midbrain dopaminergic and serotonergic neurons. However, the duration of significant changes of DA and 5-HT levels in the lesioned rats was prolonged compared to sham-operated rats. These findings are supported by the Western blotting results showing that up-regulated GluR1 subunit-containing AMPA receptors in the RMTg of the lesioned rats enhance the response of the GABAergic neurons to (S)-AMPA and NBQX, and then increases the duration of (S)-AMPA and NBQX action on DA and 5-HT release in the mPFC.

In conclusion, the results of the present study indicate that unilateral 6-OHDA lesions of the SNc in rats enhance AMPA receptor-mediated excitatory transmission in the RMTg, with findings including increased the firing rate of the GABAergic neurons and release of glutamate, and up-regulation of GluR1 subunit-containing AMPA receptors, which prolongs the duration of (S)-AMPA and NBQX action on the firing rate of GABAergic neurons in the RMTg and release of DA and 5-HT in the mPFC. From these findings, the present study provides an evidence for further understanding involvement of the RMTg and AMPA receptors in PD.

Conflicts of interest

All authors report no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neuint.2018.11.007>.

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