RESEARCH PAPER

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Changes in D_4 and $GABA_A$ subunit α3 receptors in the thalamic reticular nucleus caused by unilateral lesion of the globus pallidus

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The thalamic reticular nucleus controls information processing in thalamocortical neurons. GABAergic neurons present in this nucleus express the α_3 subunit of post-synaptic $GABA_A$ receptors, which bind GABA from globus pallidus neurons. Pallidal neurons, in turn, have dopaminergic D_4 receptors in their axon terminals. The thalamic reticular nucleus connects reciprocally with the thalamus, and it receives afferents from the brain cortex, as well as from other brain structures that have an important role in the modulation of the thalamic network. Based on the above, the purpose of this study was to assess the electrophysiological and molecular effects of unilateral lesion of the globus pallidus on the electric activity of the thalamic reticular nucleus. Two-month-old male rats were used. The right globus pallidus was lesioned with quinolinic acid. Seven days after the lesion, ipsilateral turning was registered, confirming the lesion. Afterward, electrophysiological evaluation of the right thalamic reticular nucleus' electrical activity was performed. Subsequently, mRNA expression for D_4 receptors and subunit α_3, as well as protein content were assessed in the right reticular nucleus. Pallidum lesion caused an increase in firing frequency and decreased firing bursts of reticular neurons. In addition, dopaminergic D_4 mRNA, as well as protein increased. In contrast, GABAergic $GABA_A$ subunit α_3 expression was suppressed, but protein content increased. These results show that the globus pallidus regulates firing in reticular neurons through D_4 receptors and subunit α_3 of $GABA_A$ receptor in the reticular nucleus of the thalamus.

Key words: thalamic reticular nucleus, globus pallidus, thalamocortical pathway, D_4 receptors, $GABA_A$ α_3 subunit

INTRODUCTION

The thalamic reticular nucleus (TRN) is a cluster of GABAergic neurons surrounding the thalamus (Florán et al., 2004; Sun et al., 2012; Ahrens et al., 2015; Hou et al., 2016; Villalobos et al., 2016). Reticular neurons do not project directly to the cerebral cortex, instead, they send GABAergic projections to different nearby thalamic nuclei, thereby inhibiting their activity, due to its key location between the cortex and the thalamus. All thalamic-cortical (TC), cortical-thalamic (CT),

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and reticular-thalamic (RT) projections constitute a unified and topographically organized circuit (Lam and Sherman, 2011). Furthermore, seven sectors have been identified in the TRN: motor, auditory, visual, somatosensory, limbic, gustatory, and visceral (Shosaku & Sumimoto, 1983; Shosaku et al., 1984; Kimura et al., 2012; Clemente-Perez et al., 2017; Crabtree, 2018). Many TC and CT neurons innervate a single GABAergic neuron in the TRN which, in turn, sends projections to many TC neurons (Pinault, 2004; Pratt & Morris, 2015). The large receptive fields for the TRN and its inhibitory cells have led to the idea that it has an integrative role, since each sector of the TRN receives projections from the corresponding cortical and thalamic neurons, projecting back to the thalamic nucleus that innervates it. Also, it is possible that this nucleus communicates to the cortex and the striatum through the circuit formed by the TC-RT-CT projections in the reticular neurons that can inhibit and temporarily interrupt neuronal activation in some thalamic nucleus located in the vicinity. These interactions between pairs of thalamic nuclei are reciprocal and modulate the transmission of information through the thalamus to the cerebral cortex, the striatum, and other structures of the basal ganglia (Crabtree, 2018).

The TRN receives not only glutamatergic projections from the thalamus and cortex, but also GABAergic projections from the globus pallidus (GP) (Villalobos et al., 2016), and dopaminergic projections from the pars compacta substantia nigra (SNc). However, SNc neurons do not synapse directly with TRN neurons, but rather with GABAergic pallidal neurons by axo-axonic synapses (Govindaiah et al., 2010).

The TRN nucleus has two types of neural responses, the tonic mode, and low-threshold bursts, defined by the polarization state of the cell membrane. These two responses are related to the way in which the transmission of information between the thalamus and the cortex occurs. Under depolarized conditions, cells fire in a tonic mode, generating individual action potentials of variable frequency, while in states of membrane hyperpolarization, neurons respond through low-threshold bursts. The firing mode depends on the resting membrane potential; when it is around -65 mV, firing is tonic, but when membrane voltage is lower than -65mV, firing is in bursts (Bazhenov et al., 1999; Pinault, 2004; Byoung-Kyong, 2010; Pratt & Morrison, 2015). Membrane hyperpolarization activates low-threshold Ca^{2+} channels, also called T-type channels (Bal & McCormick, 1996), the activation of which allows entry of Ca^{2+} and membrane depolarization, causing a plateau, the duration of which ranges from 100 to 200 ms. Tonic spikes are responsible for subsequent and detailed processing of select relevant information from thalamic neurons to their respective cortical association area. The burst mode responds to information deviation to primary cortices and redirects the information to association areas for memory processes and to the primary memory cortex to remember the information (Guido & Weyand, 1995).

Although the presence of GABAergic projections from the GP to the TRN is known, their role in the activity of reticular neurons has not been deeply analyzed (Asanuma & Porter, 1990; 1994; Cornwall et al., 1990; Hazrati & Parent, 1991; Gandia et al., 1993). There are a few studies addressing the pallidal-reticular pathway (Pazo et al., 2013). It has been reported that activation of GP predominantly diminishes the spontaneous TRN neurons firing rate, while its inhibition increases their firing rate (Villalobos et al., 2016). Some studies indicate the presence of a high number of D_4 receptors within the TRN, which are located, according to anatomical, immunohistochemical, electrophysiological, and pharmacologic studies, in the presynaptic buttons of pallidal axon terminals (Mrzljak 1994; Defagot et al., 1997). Thus, GABA release from pallidal neurons, mediated by depolarization, is modulated by dopaminergic receptors responding to dopamine (DA) from striatal neurons (Shin et al., 2003; Florán et al., 2004; Gasca-Martinez 2010; Govindaiah et al., 2010; Erlij et al., 2012). Dopaminergic innervation of the thalamic reticular nucleus originates from the substantia nigra pars compacta (SNc) (Gasca-Martínez et al., 2010; Ferrarelli & Tononi, 2011; Pazo et al., 2013). In summary, the activation of the GP inhibits the spontaneous activity of TRN neurons through GABAergic terminals. In turn, dopaminergic neurons from the SNc inhibit GP GABAergic transmission to the TRN *via* presynaptic D_4 dopaminergic receptors, thus activating TRN neurons, which then inhibit thalamic relay neurons, originating a dysfunctional activity in the passage of information to the cerebral cortex (Shin et al., 2003; Gasca-Martinez et al., 2010; Govindaiah et al., 2010; Pazo et al., 2013; Pratt & Morris, 2015).

It is known that the majority of D_4 receptors are present in GABAergic collateral projections going from the GP to the TRN, modulating GABA release (Erlij et al., 2012; Conde-Rojas et al., 2020). D_4 receptors are coupled to Gαi/o proteins, which inhibit adenylate cyclase and cAMP production (Lindgren et al., 2003; Beaulieu et al., 2011). $GABA_A$ receptors are part of ligand-gated chlorine channels and are responsible for rapid synaptic inhibition in the brain. In mammals, they are pentameric, with different combinations of 17 subunits. There is a great variety of subtypes of $GABA_A$ receptors in the brain, and are responsible for the functional diversity of those re-

ceptors. In rodent brain it seems to comprise at least 12 subunits: α1-6, β1-3, γ1-3 (Pirker, 2000; Chen, 2001; Bollan et al., 2003). The most abundant subunits in adult rat brain are α1-6, β2, and γ2, which are present in 60-90 % of GABA receptors; α2, α3, α5, β3 are moderately abundant, with 15-30% present in the GABA receptor (Fritschy, 1995). Specifically, the pentameric conformation of the subunits that comprise the $GABA_A$ receptor found in reticular cells are: α1 and α3, two β3, and one γ (Pirker, 2000). Electrophysiological and immunohistochemical studies show the presence of the α3 subunit in $GABA_A$ receptors, which is the main subtype expressed in the reticular nucleus of the thalamus, in contrast to other nuclei (Yee et al., 2005). The α3 subunit of the $GABA_A$ receptor is important for the function of chlorine channels in the TRN neurons, as the activation of $GABA_A$ receptors increases the passage of chlorine ions to induce neuron hyperpolarization, thus activating T-type Ca^{2+} channels, which are necessary for the generation of firing bursts (Liu et al., 2007; Mozrzymas et al., 2007). Consequently, deficiency of the α3 subunit disturbs reticular neurons response to pallidal neurons, decreasing generation of firing bursts (Yee et al., 2005; Liu et al., 2007). Therefore, the aim of this study was to evaluate, the effects of a unilateral lesion of the GP on the electrical function of the TRN by using electrophysiological and molecular approaches.

METHODS

Animals

Two-month-old, male Wistar rats (n=40), weighing 180-220 g, with free access to water and food were used. They were divided in two groups: control (n=20) and lesion (n=20). Animals were handled according to the regulations approved by the Animal Care and use Committee of the National Polytechnical Institute, School of Medicine Animal Care Facility. Num. ESM--CICUAL-01/12-10-2016.

Fig. 1 shows the experimental design.

Stereotaxic surgery (unilateral lesion of the right globus pallidus)

Animals were anesthetized with xylazine/ketamine (8 mg/kg + 70 mg/kg ip), placed on a two tower David Kopf stereotaxic device, model 902 (Tujunga, CA, USA). Supplementary doses of anesthesia were given (50-70 mg/kg) if/when the corneal reflex was present. A thermal pad and a rectal thermometer (Frederick Haer & Company, Bowdoin ME, USA) were used to monitor and maintain body temperature at 37.5°C.

For lesion of the right GP, a 30 G caliber cannula was placed with the following coordinates: antero/posteri-

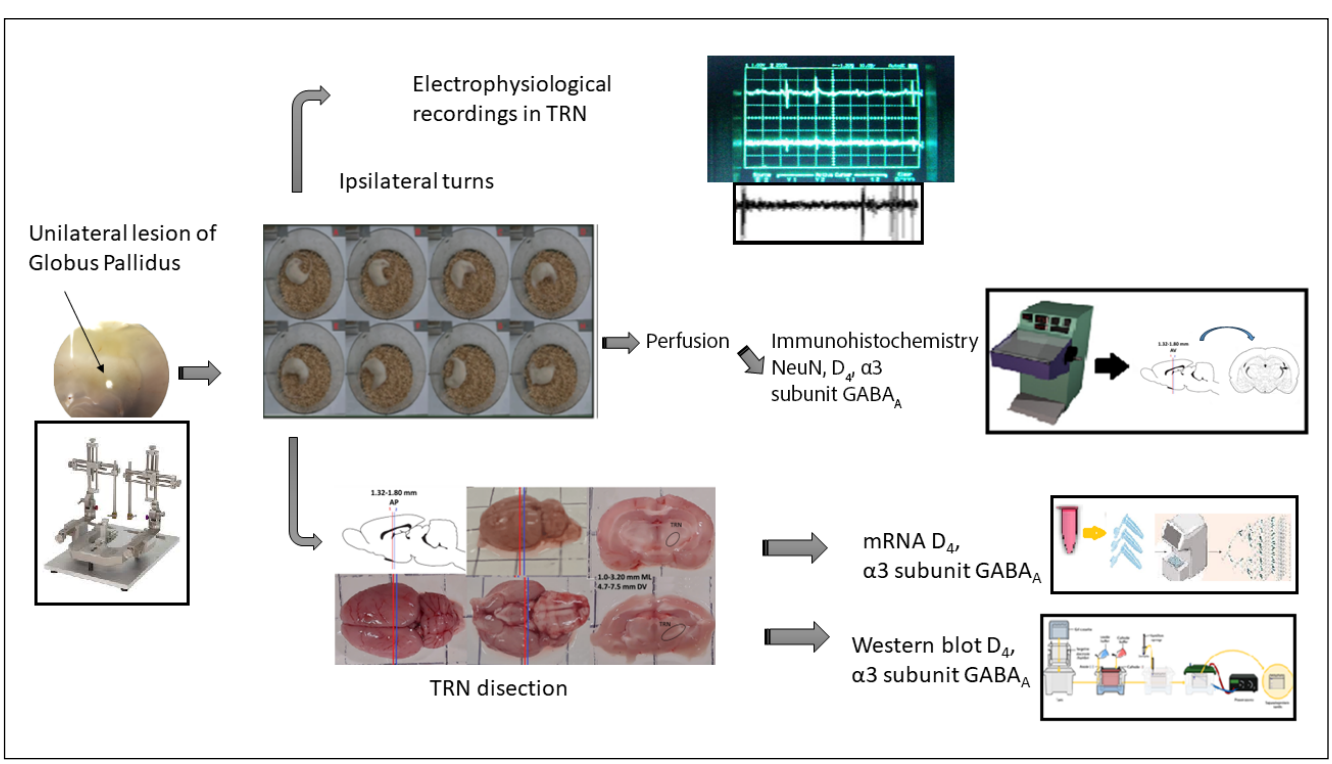

Fig. 1. Experimental design.

or (AP) -0.84 mm from bregma, medial/lateral to midline (ML) 3.0 mm, and dorsal/ventral from dura (DV) 6.5 mm (Paxinos and Watson, 2007). 200 nL of quinolinic acid (240 nmol/μL) were applied for a 4-min infusion period. The cannula was maintained in place for 5 min to allow for adequate diffusion of the neurotoxic substance. Quinolinic acid was dissolved in a NaOH (4M) solution, adjusted to a 7.4 pH using a HCl (1M) solution.

Seven days after the lesion by quinolinic acid, the lesion of the GP was assessed by inducing ipsilateral turns with apomorphine (0.1 mg/kg, i.p.). Only those rats presenting more than three turns per min were used for the evaluations. Histological analysis indicated that approximately 80% of cells in the GP died due to quinolinic acid.

NeuN immunoreactivity

One week after the lesion, four rats were euthanized with an overdose of xylazine/ketamine (24 mg/kg + 210 mg/kg, Procin®/Anesket® Vet). Afterward, they were transcardially perfused with a fixative solution (4% paraformaldehyde in PBS 0.1M, pH 7.4). Brains were extracted, stored, and fixed in the same solution for 3 h. Subsequently, brains were dehydrated in 5% and 30% sucrose solutions for 12 h, then frozen and stored at -70°C. Coronal cuts (30 μm) were obtained, 2 series of 20 slices were collected and stored in a 20% sucrose solution at -20°C. Samples were thawed and washed with PBS (0.1 M, 10% fetal calf serum and 0.5% triton). Anti-NeuN primary antibody (ab104225, Abcam), anti-rabbit secondary antibody (lsab System-hrp kit; DAKO, CA, USA), and ABC peroxidase complex were diluted in PBS with 1% fetal calf serum and 0.125% triton. Incubation with anti-NeuN primary antibody (1:1000) was carried out for 48 h; incubation with anti-rabbit secondary antibody (1:1000), and the ABC peroxidase complex was for 2 h. After incubations, each slice was washed 3 times with PBS for 5 min. Reveal was made using 3.3 mL of amino benzidine (DAKO, CA, USA) 0.05% and H_2O_2 0.01%. Finally, the sections were dehydrated in increasing alcohol concentrations, followed by xylol, and covered with DPX resin (Entellan™ new) for mounting. The left hemisphere (no lesion) was considered as control; right hemisphere contained the injured the GP. NeuN immunoreactivity was evaluated in 4 sections per animal.

Electrophysiological recordings

To evaluate unitary cell recording in neurons of the TRN, 3 control animal and 3 animals with unilateral lesion of the GP were anesthetized with xylazine/ketamine (8 mg/kg + 70 mg/kg). A 3 mm burr hole was drilled to allow for neuronal recordings. Once the duramater was visible, we proceeded to remove it using a fine hook. A recording electrode was placed in the TRN; according to Paxinos and Watson (2007), the coordinates for the location of the electrode were: 1.3-1.6 mm AP from bregma, -2.1 mm ML, 5.2-6.5 mm DV into the thalamic reticular nucleus. Borosilicate pipettes (Frederick Haer & Company, Bowdoin ME, USA) made using a puller (Pull-100 WPI, Sarasota, FL, USA) filled with a hyperosmolar $NaCl_2$ M solution and pontamine blue 1% (resistance 4-8 mΩ).

Extracellular signals were amplified 10^3x and filtered using a differential filter between 300 and 3000 Hz (DAM-80 WPI, Sarasota, FL, USA). Signals were previously processed online using a window discriminator (WPI-121, Sarasota. FL, USA), to evaluate the quality of single-unit isolation. Subsequently, signals were stored in a PC, and analyzed offline with the INF-386, program developed by Soto and Vega (1987), and custom MATLAB scripts (The MathWorks Inc., Natick, MA, USA) for peak analysis. Neurons with a stable basal discharge for 5 min were recorded. The interspike interval (ISI) (Weyand et al., 2001), which determines the presence of bursts, and the burst patterns were analyzed to establish whether the lesion of the GP modified the firing frequency of reticular neurons. Four neurons with a stable signal were recorded, one per track to carry out experiments. In total, 12 neurons per group were recorded. The coefficient of variation was considered as the ratio between the standard deviation and the mean of ISI distribution, according to criteria established by Weyand et al. (2001).

At the end of each experiment, a lethal dose (150 mg/kg ip) of pentobarbital was given. Then, a dissection by layers was done up to where the heart was visible. Before finely cutting into the right atrium, the left ventricle was injected with 20 ml of a 4% formaldehyde saline solution. The brain was removed and placed in a 4% formaldehyde solution overnight. Coronal slices (20 μm) were stained with safranine to observe the trajectories of the recording electrodes.

Thalamic reticular nucleus dissection

Animals (n=6, each group) were euthanized by decapitation, brains were removed from the skull and placed on a Petri dish on ice. The cut was made at 7.68-7.20 mm interaural, and -1.32 to -1.80 AP from bregma (Paxinos & Watson, 2007), to glimpse the tissue to be removed. Subsequently, a small spoon was introduced at the coordinates 1.0- 3.20 mm ML, and 4.7-7.5 mm DV (Paxinos & Watson, 2007). Using a leck-

on spatula, the tissue was extracted from each brain of the control and lesion; TRN was isolated and stored in 1 mL Eppendorf tubes at -80°C until assay.

Determination of mRNA expression in $GABA_A$ receptor subunit α3 and in dopaminergic D_4 receptors

Total RNA was isolated using isolating reagent TriPure (Roche, Indianapolis, IN, USA). Complementary DNA was synthesized with a single strand using the kit for reverse transcription ImProm II (Promega Madison, WI, USA.) following the manufacturer's instructions. $_c$DNA was amplified using SYBR Green Master Mix (Roche Molecular Biochemicals, Mannheim, Germany) and the following primers were used:

Target gene	Primer sequence (5′ – 3′)	Amplicon size (pb)
$GABA_A$ α3	Forward GCTCCAGTGCTTCTTCAAC Reverse TGATAGCGGATTCCCTGTTC	196
D_4	Forward GATGTGTTGGACGCCTTTCT Reverse AAACTCGGCATTGAAGATGG	154
β-actin	Forward GTGGGTAATGGGTCATAGGA Reverse AGCGCGTAACCCTCATAGAT	380

Reactions were measured real time in a Rotor-Gene (Corbett Life Science, Concorde, NSW, Australia). RT-PCR was done using the following cycle conditions: pre-incubation and denaturation at 95°C for 10 min. The threshold cycles (Ct) were measured in duplicate in separate test tubes. The integrity and purity of the amplified products were demonstrated through electrophoresis in a 2% agarose gel. The fusion curve was analyzed at the end of the amplification following the SYBER Green Kit conditions indicated by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). Every assay included a negative control for each gene. The values for ΔCt were calculated in each sample for each gene of interest as follows: Goal Ct – reference Ct (mRNA was stable throughout all experiments). The relative changes in the expression levels of a specific gene (ΔΔCt) were calculated as the ΔCt of the test group minus the ΔCt of the control group, and they were presented as $2^{-\Delta\Delta CT}$.

Content of D_4 receptor and $GABA_A$ α3-subunit

The right TRN was obtained from control (n=6) and unilateral lesion of the GP (n=6) animals and maintained at -80°C until assay. The tissue was minced in 200 µL ice cold Lysis (T-Per tissue protein reagent Thermo Fisher Scientific, Cat 78510, 0.2 M DTT (1, 4-Dithiothreitol), 0.001M PMSF (Phenylmethylsulfonyl fluoride), 0.001M NaF (Sodium fluoride), 0.2 M Na_3VO_4 (Sodium orthovanadate), protease inhibitors: Complete Mini Protease Inhibitor Cocktail (Roche Molecular Biochemical, Indianapolis, IN, USA). Tissue was homogenized using a dunce homogenizer. After incubation at 4°C for 15 min, samples were centrifuged at 12 000 × g for 10 min at 4°C. Supernatants (total protein extract) were transferred to a new tube and stored at -80°C for further use. Protein concentration was determined using the NanoDrop Spectrophotometer One C with a spectral range of 280 nm (Cat No. ND-ONEC, Thermo Fisher Scientific, USA). For western blot analysis, 200 µg of protein was loaded on a 10% SDS-polyacrylamide gel at 120 V for 90 min in a Mini-Protean ll Cell (Bio-Rad, CA, USA) along with a low-range marker standard (SeeBlue Plus2/500, Cat LC5925, Invitrogen.). Proteins were transferred to a 0.45 µm PVDF membranes (PVDF Transfer Membrane; GE Healthcare, Buckinghamshire, UK) embedded in transfer buffer (25 mM Tris-buffered saline, 190 mM glycine, 20% methanol) at 120 V at 4°C for 90 min by using the Protein Transfer System (Mini-Transblot MCA, Bio-Rad). The membranes were blocked with nonfat dried milk 5% dissolved in TTBS (137 mM NaCl, 20 mM Tris-buffered saline and 0.1% Tween 20, pH 7.6) for 30 min at room temperature. Later, the membranes were incubated overnight at 4°C with primary antibodies (diluted 1:1000) against anti-$GABA_A$ R α3 subunit, 1:500 (Cat No. NB 100-61096, Novus Biological), anti-D_4R, 1:100 (Cat No. NBP1-00779, Novus Biologicals), respectively. After three TTBS washes (10 min/each), membranes were incubated with horse anti-mouse IGG secondary antibody (Cat No. PI-2000-1, Vector Laboratories, CA, USA) at a 1:10,000 dilution for 2 h at room temperature. Membranes were washed again with TTBS three times (10 min/each). Proteins were visualized using a chemiluminescent detection kit Clarity Western ECL Substrate (contains Hydrogen Peroxide (H_2O_2) reagent and Luminol/Enhancer Reagent. Cat. No. 1705060, Bio-Rad, CA, USA) according to the manufacturer´s instructions. Bands were analyzed by using ImageJ software (developed by National Institutes of Health) for densitometric data. Stripping buffer (Tris-HCL 0.5 M, pH 2.0) was used on the membranes for later incubation with monoclonal anti β-actin peroxidase antibody (Cat No. A3854-200 UL, Sigma-Aldrich, USA) as protein loading control. The protein specific signal ratio was compared with actin, the internal standard.

Immunoreactivity

In other groups of animals (n=3, each, control, and lesion), one week after the injury, the rats were sacrificed with an overdose of xylazine/ketamine (24 mg/kg + 210 mg/kg, Procin/Anesket Vet) and transcardially perfused with a fixative solution of 4 % paraformaldehyde in a PBS buffer at 0.1 M at pH 7.4. Brains were extracted, stored, and fixed in the same solution for 3 h. Subsequently, they were dehydrated in 5% and 30% sucrose solutions for 12 h in each case, frozen and stored at -80°C. 20 µm coronal sections were obtained with a cryostat. Two series of 20 sections were collected and stored in a 20% sucrose solution at -20°C. Samples were thawed with PBS, 0.1 M, then incubated in a blocking solution for 20 min (0.1 M PBS, 20% fetal calf serum and 0.25% triton). The primary anti $GABA_AR$ α3 subunit and anti D_4R antibody (Nobus Biologicals), the anti-rabbit secondary antibody (Santa Cruz Biotechnology) and the ABC peroxidase complex were diluted in PBS +1% and fetal calf serum and 0.125% triton. The incubation for the primary anti GABA AR α3 and anti D_4R antibodies (1:500) was carried out for 24 h and the incubation with anti-rabbit secondary antibody (1:1000) and the ABC peroxidase complex was for 1 h. After incubations, each sample was washed 3 times with PBS for 5 min. To reveal, 3.3 mL of aminobenzidine 0.05% and H_2O_2 0.01% were used. The sections were dehydrated in increasing concentrations of alcohol, rinsed, and covered with DPX resin (Entellan™ new) for mounting.

Statistical analysis

Data were expressed as average values with their respective standard error of the mean. Neuronal firing rates from lesioned *versus* control rats, as well as the ISI, changes in the expression of mRNA for D_4 and α3 subunit, protein content, and immunohistochemistry were analyzed using Student's t-test. Statistical analysis was carried out with GraphPad Prism ver. 6 software. For mRNA, Western blot, and immunohistochemistry data, a value of $p \leq 0.05$ was considered statistically significant, and $p \leq 0.001$ for burst pattern and ISI.

RESULTS

Immunoreactivity to NeuN

Relative NeuN immunoreactivity showed that, compared to control (left GP) (Fig. 2A), unilateral lesion in the right GP decreased the number of NeuN-positive cells (t_{17}=39.33, P<0.0001) (Fig. 2B). The percentage of cells remaining in the right GP was close to 23%, which confirms that most of the pallidal neurons were destroyed with quinolic acid (Fig. 2C).

Electric activity

Seven days after the lesion of the right GP, the turn test induced by apomorphine (0.1 mg/kg i.p.) was performed in the control group and in the lesion group. Three days after the turn test, electrical recordings of the thalamic reticular nucleus were performed (n=12 neurons, each group). In the control and lesioned groups, two types of neuronal firings were observed in thalamic reticular neurons: spikes, and firing bursts (Fig. 3A, 3D). An amplification of the continuous signal was made to differentiate the electrical activity and identify the firing bursts of TRN neurons. A tonic regime refers to periodic firings. The temporal pattern to determine the presence of a burst is a minimum of two consecutive spikes, preceded by a silence of at least 50 ms, with a maximum of 6 ms between spikes (Weyand et al., 2001). As shown in Fig. 3B, three consecutive spikes are observed, indicating a firing burst. In the animals with a lesioned GP, neurons showed a higher frequency of spontaneous firing compared to the control group (Fig. 3C). In control animals there were 1.417 ± 0.1486 spikes/s compared to 34.760 ± 1.148 spikes/s in lesioned animals (t_{22}=28.14, P=0.0001). When amplifying an area of the recording, both spikes and bursts were observed clearly (Fig. 3B, 3E). In controls, for each neuron recorded, 80-85 of 100 firings recorded were bursts. In the animals with a lesioned GP, only 35-38 firings out of 100 were bursts, the rest were tonic firings. A comparison between the two groups as to the interval between spikes, and the time between two consecutive spikes (ISI) was also made. This analysis showed a greater number of bursts in the TRN from control animals than in that of the lesioned animals (Fig. 3F). In control animals, ISI in control animals was 0.868 ± 0.036, compared to 0.342 ± 0.605 (t_{22}=12.5, P=0.0001).

mRNA expression and immunoreactivity for D_4 and α3 subunit of the $GABA_A$ receptors

Three days after the turn test induced by apomorphine, the thalamic reticular nucleus was removed to analyze dopamine D_4 receptors and subunit α3 of $GABA_A$ receptors by RT-PCR. The analysis of D_4R mRNA in the TRN showed a higher relative expression in the animals with the right GP lesioned, compared to the control group (t_4=7.15, P=0.002) (Fig. 4A).

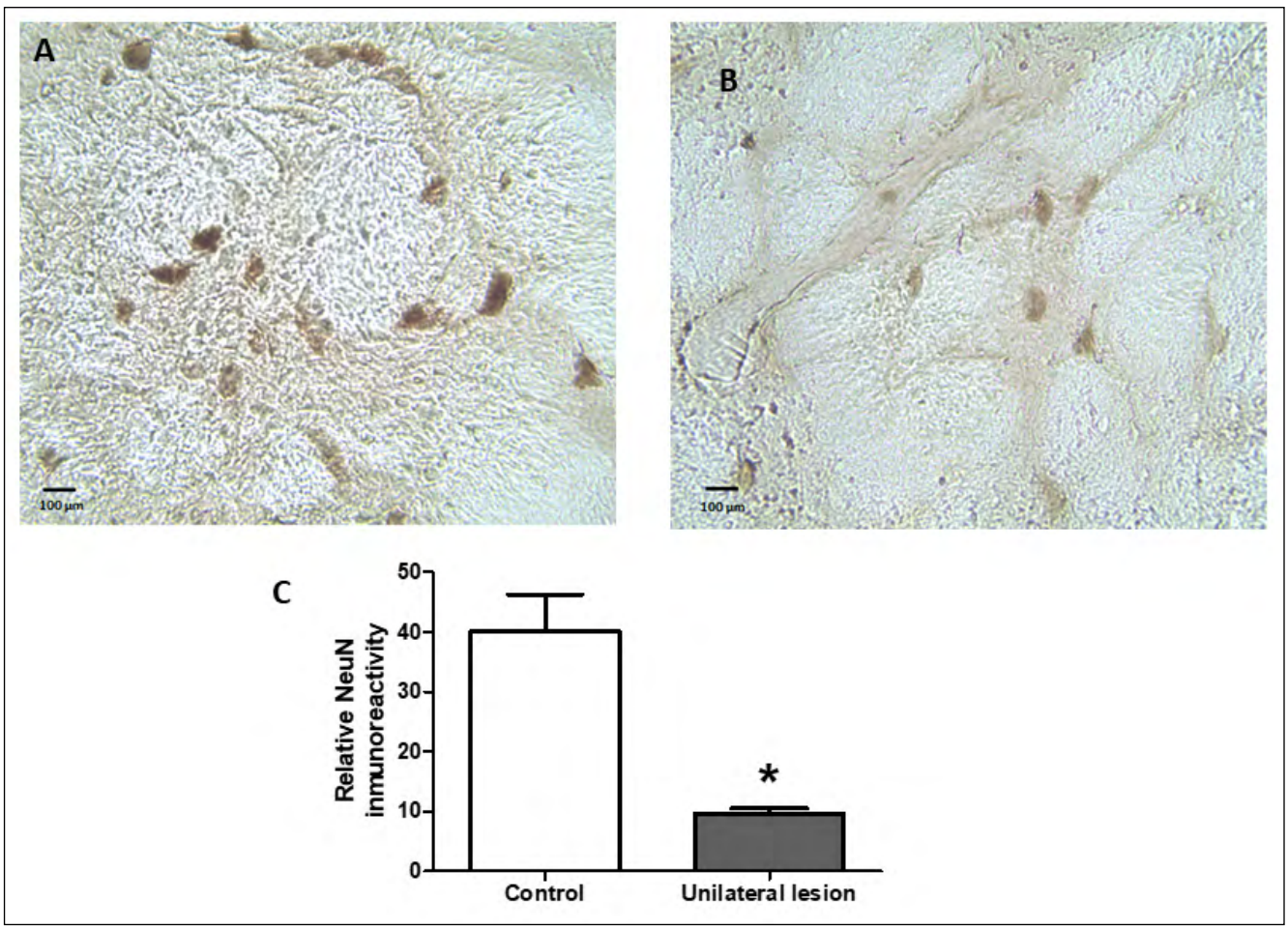

Fig. 2. Anti-NeuN immunoreactivity in the GP. Photomicrographs showing NeuN-positive neurons of control, left GP (A). Notice that the unilateral lesion (B) with quilnolinic acid administration induced neuronal loss in the right side GP. Magnification 400 x, scale bar=100 µm. (C) Anti-NeuN immunoreactivity in left, not injured, and right lesioned GP. Data shown as mean ± standard error. Student t-test. * p<0.05 compared to the control. (n=4).

Regarding mRNA relative expression of subunit α3 of $GABA_A$ receptors, it showed the opposite to that observed in dopaminergic D_4 receptors. There was lower mRNA expression in the group with unilateral lesion of the right GP compared to the control group (t_4=6.94, P=0.002) (Fig. 4B).

In other groups of animals (control and GP lesion) sections of TRN were analyzed at 200x. The label in the control group animals was relatively low, compared to that in animals with lesioned GP (Fig. 5A). The relative intensity of dopamine D_4 receptors in the TRN of lesioned GP animals showed higher intensity, with respect to control animals (t_6=3.82, P=0.008) (Fig. 5B).

Immunohistochemistry in coronal sections of TRN shows that in animals with the right-side GP lesion, the label for α3 subunit of the $GABA_A$ receptor is higher than in control animals (Fig. 6A). The relative intensity of the label for α3 subunit was higher in animals with the lesioned right-side GP (t_8=6.52, P=0.0002) (Fig. 6B).

Content of D_4 in TRN and α3 subunit of $GABA_A$ receptors

Regarding D_4 receptor protein content, it was higher in animals with lesioned right-side GP than in control animals (t_6=2.88, P=0.028). These results are in accordance to the immunohistochemistry analysis and mRNA for the protein, which were increased (Fig. 7).

The levels of the α3 subunit of $GABA_A$ receptors in the TRN of animals with the lesioned right-side GP was higher than in control animals (t_6=3.74, P=0.009). This is consistent with the increase observed in the immunohistochemical analysis for this subunit, but not with the decrease observed in mRNA for the protein (Fig. 8).

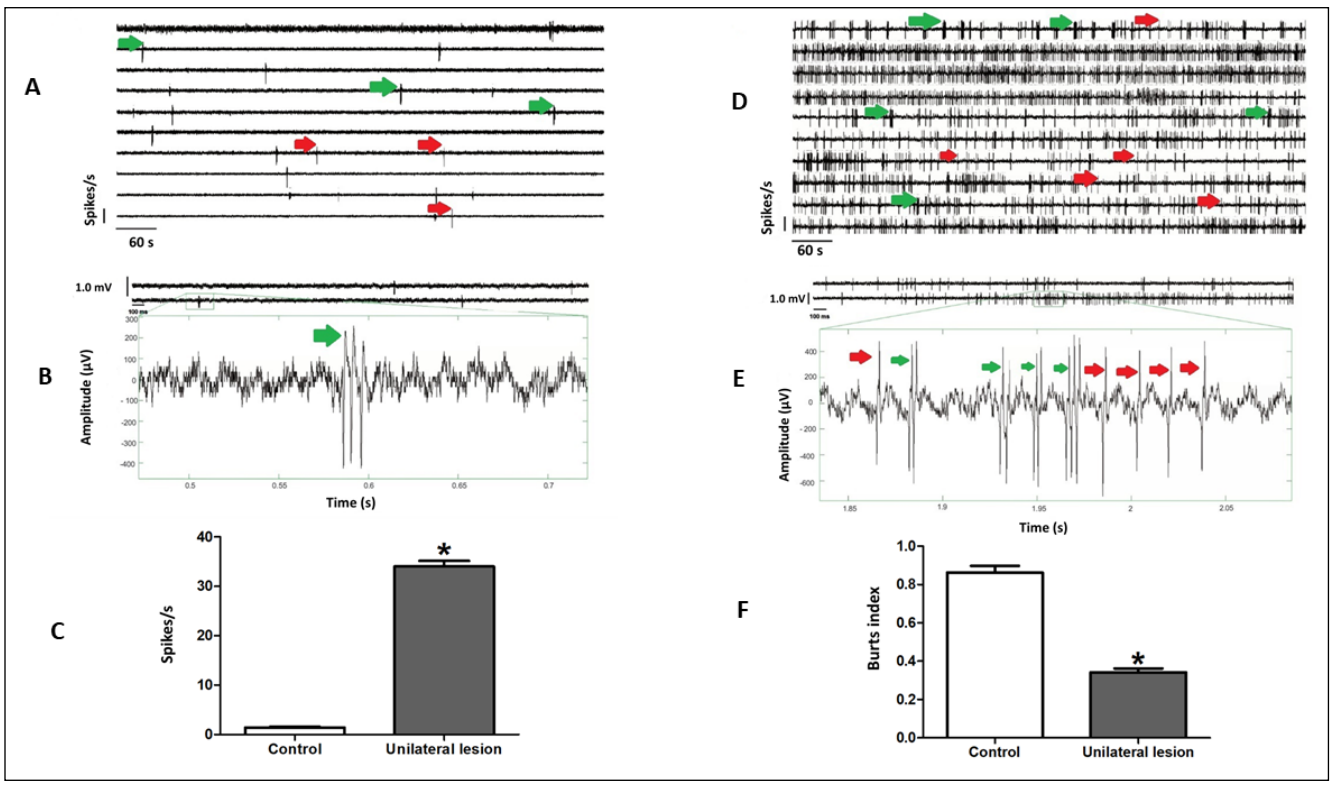

Fig. 3. (A) Electric activity of thalamic reticular nucleus neurons in the control group. Two types of firing are observed: firing bursts (green arrows) and tonic firing (red arrows). (B) Amplification of a band from the recording, showing a firing burst in controls. (C) Electric activity of thalamic reticular neurons in animals with the right GP lesioned increased. A higher tonic activity (green arrows) and lower firing bursts (red arrows) is observed. (D) Firing frequency of thalamic reticular neurons in rats with the right table lesioned. (E) Amplification of a band from the recording showing tonic and firing bursts with lesioned GP. Periodic spikes indicate tonic firings, whereas consecutive spikes indicate firing bursts. (F) Burst index in thalamic reticular neurons in control and rats with lesion in the right GP. Data shown as Mean ± Standard Error. * p<0.001 compared to control group (n=12 neurons per group).

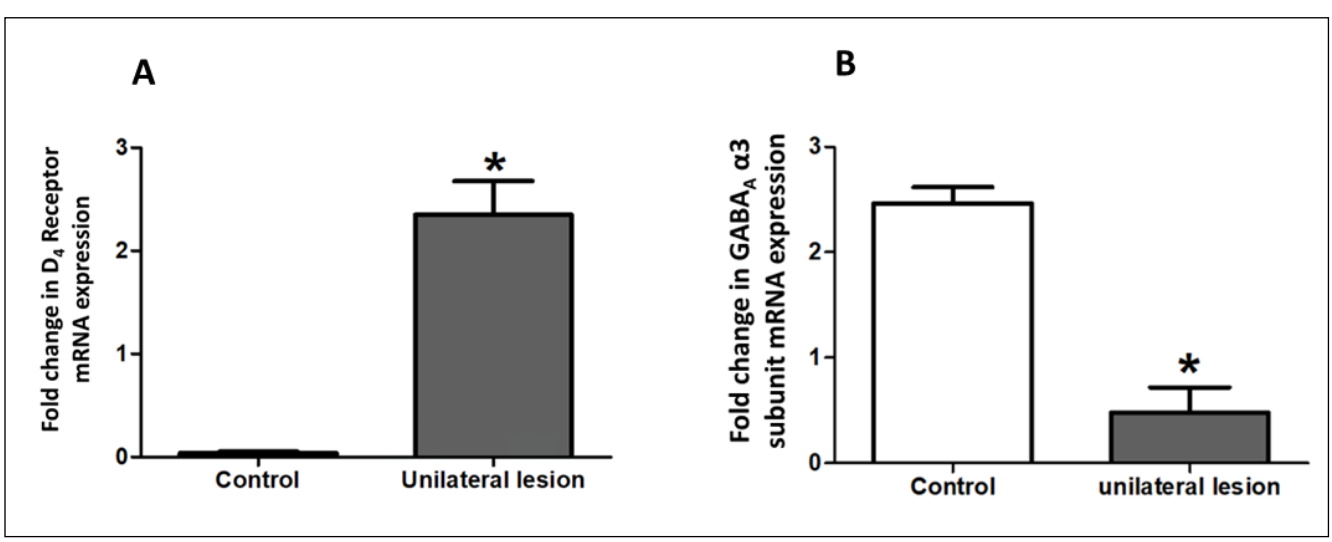

Fig. 4. (A) mRNA expression of dopamine D_4 receptor in thalamic reticular neurons. The graph compares the control group *vs.* the group with a lesion of the right GP. (B) mRNA expression of subunit α_3 of GABAergic receptors in the thalamic reticular nucleus. Data shown as Mean ± Standard Error. * p=0.002, compared to control group. (n=6 rats per group).

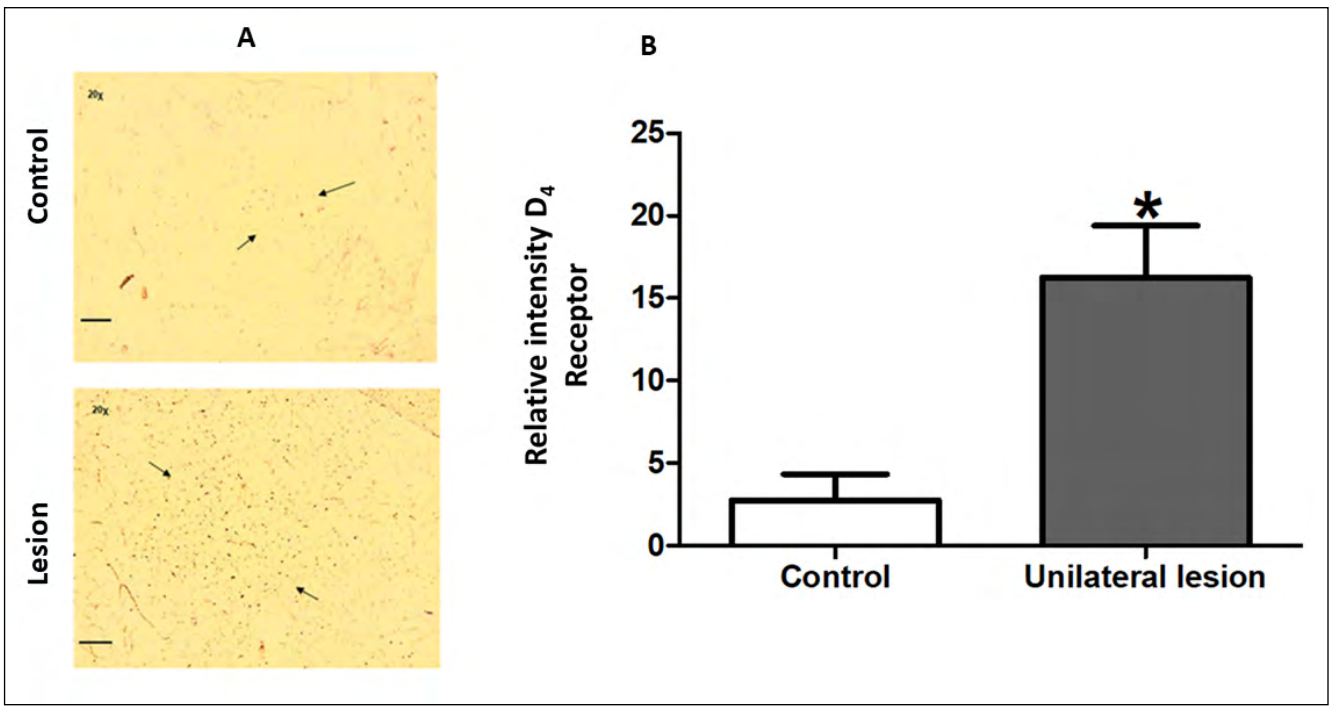

Fig. 5. Dopaminergic D_4 receptor distribution in the TRN. (A) Control and lesion group. Magnification 200 x. Positive cells for D_4 R in TRN are shown (arrows). A higher number of cells positive to D_4 receptor is observed in the lesioned group. Bar scale 10 μm. (B) Relative intensity of D_4 receptor in the TRN of control and right GP lesioned animals. The intensity is higher in lesioned animals. Data shown as Mean ± Standard Error (n=6 rats per group). * p=0.008 compared to control group.

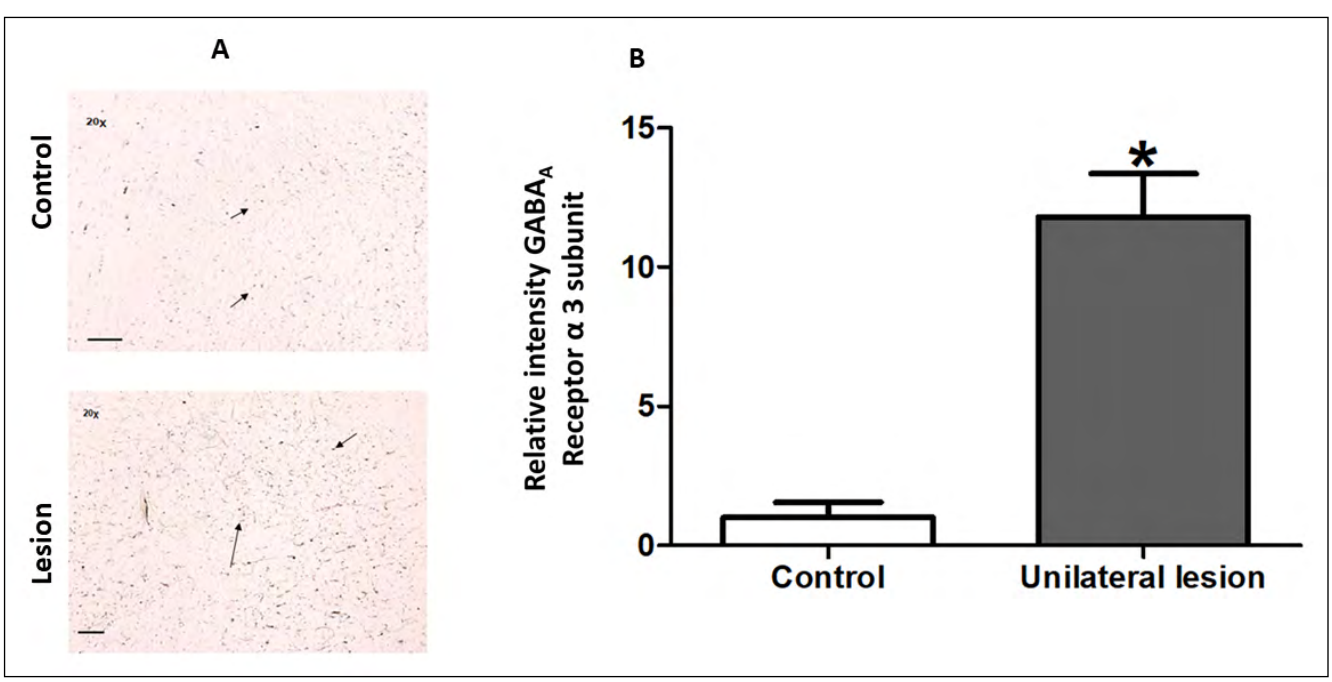

Fig. 6. Distribution of subunit α3 of $GABA_A$ receptors in the TRN. (A) control and lesion group. Magnification 200 x. Positive cells to α3 subunit are shown (arrows) in the TRN nucleus. Bar scale, 10 μm. (B) Relative intensity of α3 subunit of $GABA_A$ receptors in the TRN. The number of cells labeled is higher in the TRN of animals with the right GP lesioned than in control animals. Data shown as Mean ± Standard Error (n=6 rats per group). * p=0.0002 compared to control group.

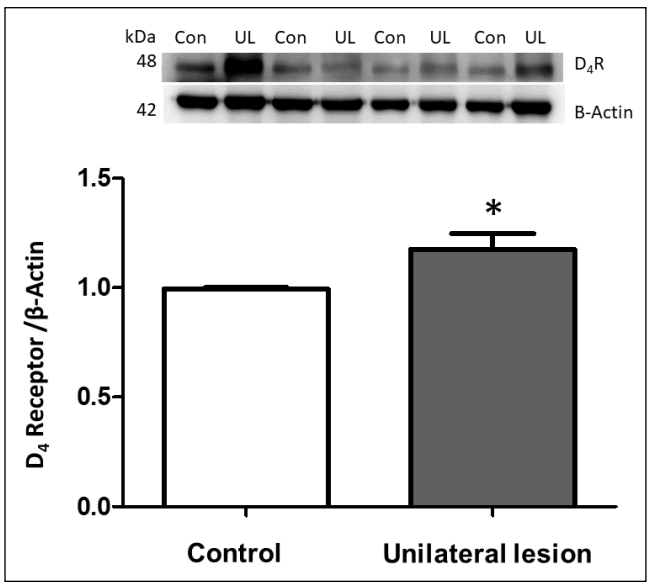

Fig. 7. Dopaminergic receptor D_4 content in the TRN of control and animals with the right GP lesioned. The content of the protein is higher in the experimental group. Data shown as Mean ± Standard Error (n=6 rats per group). * p=0.01 compared to control group.

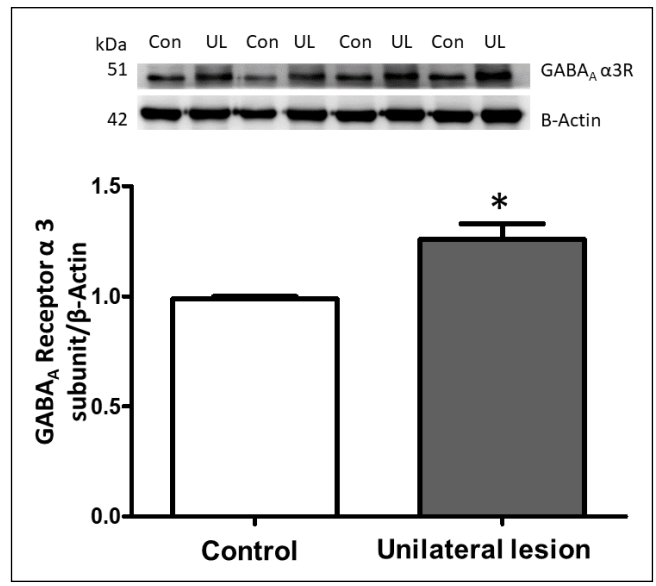

Fig. 8. Content of α3 subunit of $GABA_A$ receptors in the TRN of control and animals with the right GP lesioned. The content of protein is higher in the experimental group. Data shown as Mean ± Standard Error (n=6 rats per group). * p=0.01 compared to control group.

DISCUSSION

The thalamic reticular nucleus is a group of GABAergic neurons which is involved in multiple processes for the integration and processing of sensory, motor, attentional and cognitive information, and it is responsible for the modulation of rhythmic thalamocortical activities (Lee et al., 2007; Pinault, 2004). Reticular neurons predominantly have subunit α3 of $GABA_A$ receptors (Mozrzymas et al., 2007; Pratt & Morris, 2015; Waldvogel et al., 2017). Their firing mode is determined by the membrane potential, and is characterized by presenting two types of firings, one called tonic, which occurs when the cell is depolarized and is dependent on its interactions with the cortex and the thalamus (glutamatergic projections), the locus coeruleus (noradrenergic projections), the dorsal rafe nucleus (serotonergic projections) and the penduncular-pontine nucleus (cholinergic projections). The other type of firing is in bursts and occurs when the cell is hyperpolarized; this firing is dependent on GABAergic projections originating in the GP (Morrison & Foote, 1986; Pinault, 2004; Govindaiah et al., 2010). Axon terminals in the GP receive dopaminergic fibers from the SNc, which modulate the release of GABA by presynaptic inhibition of dopaminergic D_4 receptors (Pazo et al., 2013).

Pallidal lesions with quinolinic acid have an optimal effect, since they are specific to the internal GP (endopeduncular nucleus) in striatopallidal projections and does not cause lesions in the axons that pass through it, for example, the nigrostriatal or striatonigral pathway (Hauber et al., 1998; Lonser et al., 1999). The present results show that unilateral lesion of the GP caused an increase in firing frequency in ipsilateral TRN neurons, which may be attributed to a reduction in GABA release due to the loss of GABAergic neurons projecting from the GP, since only 20% of cells remained after the lesion. At the same time, thalamocortical, corticothalamic and brainstem nuclei projections appear to favor an increase in the number of tonic firings due to DA, 5HT and NA released by neurons coming from SNc, Raphe dorsal, and locus coeruleus, respectively (Guillery & Harting 2003).

Low GABA release could also be responsible for the reduction in firing bursts from thalamic reticular neurons. The TRN contains GABAergic neurons and is involved in multiple processes of integration and processing of sensory-motor, attentional and cognitive information, and is also responsible for the global modulation of thalamocortical rhythmic activities (Lee et al., 2007; Pinault, 2004). The α3 subunit of $GABA_A$ receptors predominates in TRN neurons (Mozrzymas et al., 2007; Pratt & Morrison, 2015), which determines the degree of inhibition or silence that the GP exerts on the cells of the TRN generating firing bursts (Liu et al., 2007). Furthermore, the main GABAergic input from the GP to the TRN is modulated by dopaminergic D_4 receptors, which are located in the pallidal-reticular axon

terminals (Gasca-Martinez et al., 2010; Govindaiah et al., 2010). So, the dopaminergic fibers coming from the SNc, modulate the release of GABA through presynaptic inhibition by dopaminergic D_4 receptors (Florán et al., 2004; Pazo et al., 2013).

Regarding the results of the relative expression of the dopaminergic D_4 receptor mRNA in the TRN, an increase was observed in the individuals of the group with unilateral lesion of the right GP, compared to control animals. Considering that the lesion of the GP was not total, but that only 80% of pallidal neurons were destroyed, it is possible that the 20% remaining neurons in the GP increased their expression and protein D_4 content, probably as a compensatory mechanism, due to the presence of recurrent collaterals from the dopaminergic projections coming from the SNc, which are more than 50% (Gasca-Martinez et al., 2010). Regarding the decrease in the relative expression of the α3 subunit of the GABAergic receptor mRNA in the TRN, this could be due to the low release of GABA, given the small number of GABAergic neurons remaining after injury (23% approximately), together with the increase in D_4 receptor, which can contribute to the inhibition of GABA release from the GP, resulting in a decrease in the α3 subunit of the GABA receptor mRNA (Graziane et al., 2009). Considering that this nucleus is innervated by dopaminergic axons originating from the SNc, and that the overexposure of neurons to a neurotransmitter causes tolerance or desensitization, changing the synthesis or degradation of the receptors, it can be speculated that the increase in dopamine D_4 receptor mRNA, and in the protein could be due to dopaminergic projections of recurrent collaterals coming from the SNc. Probably, these projections continue to release the same amount of the neurotransmitter; however, the pallidal lesion reduces the number of neurons receiving dopamine, and an increase in the number of D_4 receptors could be a mechanism to compensate for the reduction in GABAergic transmission after pallidal lesion. The decrease in the α3 subunit of GABA receptor mRNA in the TRN contrasts with the immunohistochemistry data and the amount of protein in the TRN. Possibly, this contrast could be due to interference RNA (iRNA), as a mechanism of post-translational silencing of specific genes, consisting in the degradation of specific mRNA, or by post-translational processes, such as methylation, acetylation, hydroxylation, or phosphorylation (Agrawal et al., 2003; Zhu & Palli, 2020; Mattick & Amaral, 2022). There are other possible mechanisms, such as small non-coding RNAs (snoRNA). These are predominantly required in the maturation of nuclear RNAs and ribosomal RNA. snoRNAs have other cellular functions such as transcriptional regulation, and many of them are derived from introns, non-protein-coding transcripts, with functions of RNA regulators (Nahkuri et al., 2008; Ogorelkova et al., 2009; Kishore et al., 2010).

The results of the current study show a decrease in the expression of the α3 subunit of $GABA_A$ receptor within the TRN after unilateral lesion of the GP. However, the protein content increased. This could be due to fewer GABAergic projections from the GP, leading to low GABA release from the GP to the TRN neurons, thus increasing the synthesis of $GABA_A$ receptor α3 subunit. However, tonic firing increased due to the GP lesion, which indicates that the increase in α3 subunit of $GABA_A$ does not compensate for the lack of GABA, losing the inhibition of the TRN cells by GABA, increasing tonic firing.

GABAergic projections from the GP to the TRN, can directly influence thalamocortical transmission through activity of TRN neurons (Asanuma, 1994), and TRN is the linking structure for thalamocortical and corticothalamic circuits, acting as a mediator of selective attention and an initiator of corticothalamic rhythmic activities (Zhao et al., 2020). Therefore, if the activity of the TRN becomes aberrant, or there is some defect in GABAergic neurotransmission by the neurons of this structure, this can cause the activation of the cerebral cortex and generate unusual rhythms in processing thalamocortical information.

CONCLUSIONS

The findings of the current study show that unilateral lesion of the GP increases tonic firing frequency and decreases the bursts of TRN neurons. This can be due to the decrease in pallidal neurons releasing GABA to TRN, and α3 subunit of $GABA_A$, as well as the increase in D_4 receptor expression and content, due to collaterals coming from SNc.

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