

Protective effect of Curcumin against rotenone-induced *Substantia nigra pars compacta* neuronal dysfunction

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Abstract

Rotenone is involved in the degeneration of dopaminergic neurons, and curcumin may prevent or effectively slow the progression of Parkinson disease (PD). Previous research has shown that the naturally occurring phenolic compound curcumin can reduce inflammation and oxidation, making it a potential therapeutic agent for neurodegenerative diseases. The present study involves investigation of rotenone induced histological changes in the brain areas, hippocampus using Nissl staining after 35 day of subcutaneous injection administration of rotenone in adult male rats. In this study, we investigated whether curcumin protects against rotenone-induced dopaminergic neurotoxicity in a rat model by *in vivo* electrical recording from Substantia nigra pars compacta (SNc). Curcumin treatment significantly improved electrical activity of neurons in the SNc of rotenone-induced PD model rats. The pattern of histological alterations corresponds with electrophysiological manifestations.

Introduction

Parkinson's disease (PD) is an age-related neurodegenerative disease characterized clinically as a movement disorder. Motor symptoms in Parkinson's disease are caused by the selective degeneration of dopaminergic neurons in the ventral midbrain's Substantia nigra (SN), which depletes dopamine levels in the striatum. Rotenone is a naturally occurring plant toxin, which has been developed into a widely used pesticide and insecticide. The toxicity of rotenone has been demonstrated in a number of *in vitro* (Hartley A et al.1990) and *in-vivo* (Caboni P et al.2004) studies. Furthermore, it has been demonstrated that when low doses of multiple exogenous factors are combined, synergistic neurotoxicity may occur. Rotenone's effect has been attributed to the inhibition of mitochondrial complex I (Hoglinger GU et al. 2003), the release of NADPH oxidase-derived superoxide from activated microglia (Gao HM et al. 2002) and possibly alteration of glutamate transmission (Leng A et al. 2003). As a result, novel therapies involving natural antioxidants and plant products/molecules with neuroprotective properties are being used as adjunctive therapy. Curcumin is a polyphenol and an active component of turmeric (*Curcuma Longa*), an Indian dietary spice and medicine. Curcumin exhibits antioxidant (Ruby AJ et al. 1995), anti-inflammatory (Aggarwal BB et al. 2009) and anti-depressant properties (Kulkarni SK et al. 2008), Several studies in various Parkinson's disease experimental models strongly support the clinical use of curcumin in PD (Mythri RB et al. 2012). Curcumin could also protect against rotenone induced toxicity (Qualls Z et al. 2014). Curcumin has also been suggested to potentially reduce the incidence of Parkinson's disease, as evidenced by studies showing an absence of age-related changes in nigral dopaminergic neurons in Indian populations that consume large amounts of curcumin (Darvesh AS et al. 2012). However, the link between electrophysiological characteristics and neuronal morphology has not been found yet. To investigate these issues, we recorded and classified the electrical activity of individual SNc neurons in control, Curcumin-treated, and Rotenone-treated Parkinson's disease rat models. Then, we analyzed the spike activity and the morphological changes in the SNc following a rotenone-induced degeneration. In this manner, the electrophysiological impairments could be related to the morphological alterations in the SNc in the pathophysiology of PD.

The current study aims to determine the activity of dopaminergic neurons in the SNc following rotenone injection and to estimate the neuroprotective effect of curcumin.

Materials And Methods

Drug and chemical agents

Rotenone and Curcumin were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Animals and treatments

Experiments were performed on 15 Sprague-Dawley 12–14 month-old male rats. Rats were kept under typical conditions of the laboratory vivarium. The experimental protocol satisfied the provisions of European Communities Council Directive (2010/63/UE) and was approved by the Ethics Committee of Yerevan State Medical University after Mkhitar Heratsi.

Rotenone model

The male adult albino rats randomly divided into three groups and treated as follows:

Group R) Rotenone rats with subcutaneous injection (2.5 mg/kg/day) for 5 weeks.

Group S) Sunflower oil (vehicle) rats (1ml/kg/d, IP) for 5 weeks.

Group C) Curcumin rats (Rotenone (5 weeks) + Curcumin, 200mg/kg (IP) for 5 weeks).

Morphological Study

Nissl-staining

After each electrophysiological experiment, rats were sacrificed 10 weeks after PD model induction and Curcumin treatment, and the brains were fixed in a 4 % formalin solution in phosphate buffer at pH 7.4. Serial frozen sections of the SNc were then washed twice with phosphate buffer and stained with 1 % methylene green solution. Hippocampal samples were submerged in 0.5% cresyl violet acid solution for 30min. Afterwards, samples were washed in distilled water and dehydrated in graded ethanol solutions (70%, 95% and 100%). The slices were finally cleared in xylene (twice, 5 min each). Then, the slices were coverslipped with dibutylphthalate polystyrene xylene organic mounting medium. Once dried, histological images at the level of substantia nigra and hippocampus were obtained with a digital camera coupled to a light microscope. Histological preparations were analyzed using a rat brain atlas (Paxinos, G. et al. 2007).

In vivo extracellular electrophysiology

At the completion of the 8-weeks study period all rats (weighing 220 ± 20 g) were euthanized with urethane (1.1 g/kg, i/p). Animals were immobilized with 1% dithylinum (25 mg/kg, i/p). Anesthetized and

shaved rats were placed in a stereotactic frame and artificial ventilation was used. For recording of extracellular spike activity a microelectrode (tip diameter 1–2 μm , resistance, 1.5–2.5 MW) filled with a 3 M KCl solution was repeatedly submerged into the SN, according to rat brain atlas stereotaxic coordinates (AP – 5; L \pm 2; DV + 7.5–8.0 mm) (Paxinos, G. et al. 2005). High frequency stimulation (HFS, 100 Hz for 1 sec) of the hippocampus (AP – 3.2–3.5; L \pm 1.5–3.5; DV + 2.8–4.0 mm) was performed using bipolar silver electrodes with application of rectangular impulses of current with a duration of 0.05 ms, and an amplitude of 0,6–0,8 mA. The statistical significance of the heterogeneity of interspike intervals (or spike frequency) of the pre- and post-stimulus impulse flow was analyzed by Student's t-test and Mann–Whitney U test. The average peri-stimulus time histogram (PSTH) of neurons with uniform responses was constructed based on the analysis of peri-stimulus firing rate for given populations. Intra-population variance ratio between time intervals during HFS and post-stimulation compared to baseline was calculated using Student's t-test. The statistical significance between groups was estimated according to Student's t-test.

Results

In the Group R tetanic potentiation (TP) in response to HFS in hippocampal neurons expressed 1.38 times (28.18:20.8 spike/sec), tetanic potentiation during HFS in neurons with TP PTP responses – 3.64 times.

In the Group S tetanic potentiation during HFS in hippocampal neurons with TD PTD expressed 1.2 times, tetanic depression (TD) during HFS in neurons with TD responses – 3.6 times, TD PTP responses – 3.62 times.

In the Group C tetanic depression during HFS in hippocampal neurons with TD PTD expressed 1.6 times ($M_{TT}=19.75 / M_{BE}=12.41$ spike/sec), tetanic depression during HFS responses – 4.49 times ($M_{BE}=35.23 / M_{TT}=7.84$ spike/sec).

The tetanic potentiation with TP PTP responses was observed in Group R.

Sections were photographed using an Olympus BX51 microscope in various magnifications to allow further digital processing and analysis. SNc and hippocampal images at original magnification ($\times 100$ and $\times 40$ magnification) were taken and analyzed for the frequency of degenerative cells. Nissle staining of the Rotenone cross-sections showed higher degenerative changes at 10 week in rat (Figs. 2, 3). Rats of Rotenone group showing necrotic neurons body pyknosis and cytoplasm stained darkly; rats that recieved Curcumin showing reduction on neurodegeneration induced by rotenone. Representative photomicrographs are from 5 animals per group. Injured neurons (rotenone group) were characterized by cytoplasmic shrinkage, nuclear pyknosis and hyperchromasia. Note the abundance of small, darkly stained, dead/dying neurons with remarkable shrinkage in the hippocampus. No abnormal Nissl bodies were found in the Curcumin group.

The results obtained in our study showed signs of neuronal damage at the level of the SNc in rotenone group of rats with an observable reduction in the number neurons. The Rotenone group showed

decreased Nissl granules in the perikarya and dendrites of nigral neurons (Fig. 3A). In neurotoxic models of Parkinsonism, parkinsonism-like symptoms are detectable only when markers of dopaminergic neurons in the SN fall below 20–40% of normal values (Breit S et al. 2007). Curcumin group showed a marked increase of the neuronal population in SNc. Methylene green staining indicated that SNpc showed large abundant purple Nissl granules in the perikarya and dendrites of nigral neurons (Fig. 3C-D).

Discussion

One of the main goals of our study was to see if increased excitation following rotenone intoxication causes dopaminergic cell death. To acquire supporting evidence, *in vivo* extracellular examination of SNc dopaminergic neurons is an appropriate way. Increased cholinergic input to dopaminergic neurons has been found to be a probable cause for dopaminergic cell death (Fonck C et al. 2003).

In vivo electrophysiological study was done to investigate the neuronal activity of the SN in response to HFS of hippocampus in a rat model of PD. To characterize the neuronal activity of the SNc neurons, *in vivo* extracellular recording was performed and the signals from 125 neurons were successfully collected. These neurons had a high firing rate and irregular firing pattern. Out of 125 neurons detected in the PD rats, there are 45 (R group), 40 (S group), and 40 (Curcumin) neurons.

Figure 1 shows three distinct firing patterns. When we compared the firing properties of the neuronal subtypes in the R group to the Curcumin, we discovered that the firing rates in Curcumin-treated neuronal populations are higher than in the R and S groups. Following that, we looked into the R and S groups. In comparison to the control group (S), the pathology group (R) was suppressed in the low-frequency. When the electrophysiological data was analyzed together (Fig. 1), it was discovered that the firing rate of the Curcumin group of neurons increased in response to HFS (100 Hz). We used subcutaneous dosing (2.5 mg/kg/day) to administer rotenone daily for up to 5 weeks. In the SN, there was a dose-dependent decrease in evoked neural activity and a decrease in firing strength. In the rotenone group, we found more expressed TP PTP responses than in the Curcumin group (Fig. 1). The pathological changes have been shown to have a significant impact on glutamatergic neurons. When compared to the non-lesioned side, glutamatergic neurons on the lesioned side had a distinct difference in cell appearance or quantification (Moran, R. J. et al. 2011). Glutamate-mediated excitotoxicity is thought to play a significant role in neuronal death during degenerative processes, and there is evidence that, under certain conditions, mitochondrial dysfunction may sensitize neurons to glutamate NMDA receptor-mediated excitotoxicity (Calabresi P et al. 2001). We also suggest that the mechanism of rotenone's action on the SN is excitatory. Marsden et al. (2007) demonstrated in rat slices that activation of NMDA receptors, which induces excitatory long-term depression (LTD) via AMPA endocytosis, increases the expression of GABAARs on the dendritic surface of hippocampal neurons (Marsden KC et al). One of the possible explanations for the cell-type-specific vulnerability induced by rotenone in the basal ganglia is the dopamine (DA) dependence of the rotenone-induced neurodegeneration. In fact, it is possible that the high endogenous DA levels present in both the pars compacta of the substantia nigra and the striatum render these structures selectively prone to toxicity induced by mitochondrial complex inhibition. Reduced

dopamine levels in striatum and hippocampus were found in rotenone models (Ulusoy GK et al. 2011). Dopamine neurons arising from the ventral tegmental area and SNc contribute during the formation of rewarded behaviors (Bayer HM et al. 2005). Substantia nigra pars compacta (SNc) dopamine neurons are autonomously active; that is, they generate action potentials at a clock-like 2–4 Hz in the absence of synaptic input (Surmeier DJ et al. 2005). In this respect, they are much like cardiac pacemakers. Juvenile dopamine-containing neurons in the SNc use sodium influx as the pacemaking mechanisms common to neurons not affected in PD, but the sodium mechanism remains latent in adulthood (Chan CS et al. 2007). Instead, the autonomous activity is generated by Ca²⁺ influx. The SNc dopamine neurons rely on L-type Ca(v)1.3 Ca²⁺ channels (Surmeier DJ 2007). DA neurons fire phasic bursts in response to unpredicted rewards, and their phasic firing begins to track neutral stimuli that predict those rewards. This firing characteristic of DA neurons suggests that they are highly effective at pairing neutral stimuli to unconditioned stimuli, and this property provided evidence that DA signals are a neural substrate of reward prediction (Montague PR et al. 2004). Current Parkinson's disease treatments aim to increase dopamine levels in the striatum in order to alleviate the associated motor deficits. These include dopamine precursors (levodopa), dopamine agonists (pramipexole, ropinirole), and MAO-B inhibitors (selegiline, rasagiline) (Senek M et al. 2014). However, these approaches do not provide a long-term solution because their efficacy diminishes as dopaminergic neurodegeneration progresses. The unsatisfactory effects of traditional antiparkinsonian drugs prompted the search for novel alternatives.

In the view of the hippocampal neural appearance (Fig. 2A), the relatively large cells in this region were observed to be fusiform, medium-small sized cells were round or oval with thinner dendrites. Hippocampal neurons became round or oval, with small soma following 5 weeks Rotenone intoxication.

The results obtained in our study showed signs of neuronal damage at the level of the SNc and Hippocampus in Rotenone-group animals with an observable reduction in the number neurons (Figs. 2, 3). In physiological conditions, the Nissl bodies are big and abundant, showing that the function of neuronal protein synthesis are strong; on the other hand, when nerve cells are damaged, the number of Nissl bodies will be reduced or even disappear. Intranuclear Nissl bodies of the SNc and hippocampus are lightly stained and appeared to be sparsely arranged in the Rotenone model group. However, deeper stained Nissl bodies with higher density in SNc and hippocampus neurons were found in the Curcumin group. Previous studies have revealed that the natural phenolic compound curcumin can reduce inflammation and oxidation, which makes it a potential therapeutic agent for neurodegenerative diseases. In this study, we investigated whether curcumin protects against rotenone induced dopaminergic neurotoxicity. Recent studies suggest that Curcumin can protect DA neurons from degeneration in experimental PD (Yu S et al. 2010). Besides, memory performance was also recognized to be greatly increased (Darbinyan L.V. et al. 2017, Song S et al. 2016). Another research recently found that pre-treatment with curcumin accompanied by PQ exposure to PINK1 siRNA cells showed increased mitochondrial membrane capacity and reduced apoptosis (Merwe C et al. 2017). Therefore, in PD treatment, curcumin provides strong promise. Over 70% of SNc input is GABAergic, including afferents from the rostromedial tegmental nucleus (Hong S et al. 2011), striatal patches (Fujiyama F et al. 2011) and SNr (Tepper JM et al. 1995). It also receives glutamatergic afferents from diverse subcortical

structures including STN and PPN (Morikawa H et al. 2011). Further, local somatodendritic dopamine release influences both SNc and SNr [33]. GABAergic cells fire more frequently, and evoke greater membrane depolarization (Kajikawa, Y. et al. 2011). The analysis of spike waveform duration was used to classify SNc neurons. GABAergic inputs effectively modulate the firing pattern of dopaminergic neurons in vivo. Local GABA(A) receptor blockade causes dopaminergic neurons to switch to a burst firing pattern, regardless of the original firing pattern. This is accompanied by a slight increase in the rate of spontaneous firing. GABAergic inputs from axonal collaterals of pars reticulata neurons appear to be a particular source of GABA tone for dopaminergic neurons (Tepper JM et al. 2007). Curcumin has been determined to allosterically modulate GABA receptors in a manner distinct from benzodiazepines (Liu, YM. et al. 2017). It promotes GABA-activated current in hippocampal neurons and cell lines expressing endogenous and recombinant GABAARs33, respectively (Ding, J. et al. 2014). In our experiment, after 35 days of curcumin administration, the activity of SNc neurons with depressive type TD responses tended to increase in response to HFS. Curcumin significantly increased the expression of TD and TD PTD responses in the SNc in response to hippocampal HFS compared to the control and Rotenone groups. GABAergic neuron loss was observed in a rat model of Parkinson's disease, which was also thought to be a feature of PD patients (Pienaar I. et al. 2013). It was shown that both acute blockade of D1/5 DAergic receptors (D1/5R) and chronic DA depletion abolish nigral LTD, and that activating D1/5R restores LTD expression in DA-depleted slices. Thus, impairments in DA-dependent adaptations of STN-SNr synapses observed in experimental parkinsonism (Dopamine-Dependent Long-Term Depression at Subthalamo-Nigral Synapses Is Lost in Experimental Parkinsonism (Julien Pierre Dupuis et al. 2013). SNc neurons appeared to have stronger inhibitory response to stimulation frequency and were silenced at 100 Hz in most cases. Higher prevalence of GABA synapses in SNr likely explains why SNr neurons exhibited a greater inhibitory response to electrical stimulation and following Curcumin treatment. We have previously showed that rotenone is a critical player in the host of cellular and synaptic changes (activity-dependent synaptic plasticity) induced in hippocampus by dopamine depletion (Darbinyan L.V. et al. 2017) and Curcumin protects hippocampal neurons against rotenone-induced cell death (Darbinyan L.V. et al. 2017). The above electrophysiological data show that curcumin protected PD animals against Rotenone injury, which histologically might be explained by activation of GABA neurons and signaling pathways. It was also determined that Curcumin significantly attenuated rotenone-induced dopaminergic neuronal oxidative stress-induced injury in the substantia nigra region of rats via the activation of the protein kinase B/nuclear factor erythroid 2-related factor 2 signaling pathway (Cui Q et al. 2016). Curcumin showed a neuroprotective effect against 6-OHDA-induced hippocampus neurons in rats, and the underlying mechanism promoted hippocampal tissue regeneration via activating the brain derived neurotrophic factor/Tropomyosin receptor kinase B pathway (Yang J et al. 2014).

Here, we show that Curcumin administered through the *i.p.* route provide substantial protection to SN DA neurons in a rotenone rat model of PD. Our study provides evidence for the therapy of Parkinson's disease as well as the underlying mechanism of curcumin's neuroprotective activity. Given the intricacy of molecular and neurological systems, more research is needed to pinpoint the precise process.

Declarations

Author Contributions: Study Concept and Design: L.D., K.S.,V.S. Acquisition of Data: L.D., K.S.. Analysis and Interpretation of the Data: L.D., K.S., Drafting of the Manuscript: L.D., K.S., L.H., L.M., V.S. and C.V.

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Availability of data and materials: Raw data can be provided upon request to the corresponding author.

Ethics approval and consent to participate: The experimental protocol corresponded to the conditions of the European Communities Council Directive (2010/63/UE) and it was approved by the Ethics committee of the Yerevan State Medical University after Mkhitar Heratsi.

Approval code-N4 IRB APROVAL, November 15, 2018.

Patient consent for publication: Not applicable.

Competing interests: The authors, L.V. Darbinyan, L.E. Hambardzumyan, L.P. Manukyan, K.V.Simonyan, C.A.Vasconcelos, V.H. Sarkisian declare the absence of any conflict in commercial or financial relations, relationships with organizations or persons that in any way could be related to the study, and also in interrelations of the co-authors. As well as all present authors agree with all norms and requirements demanded by the MOLE Journal.

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Figures

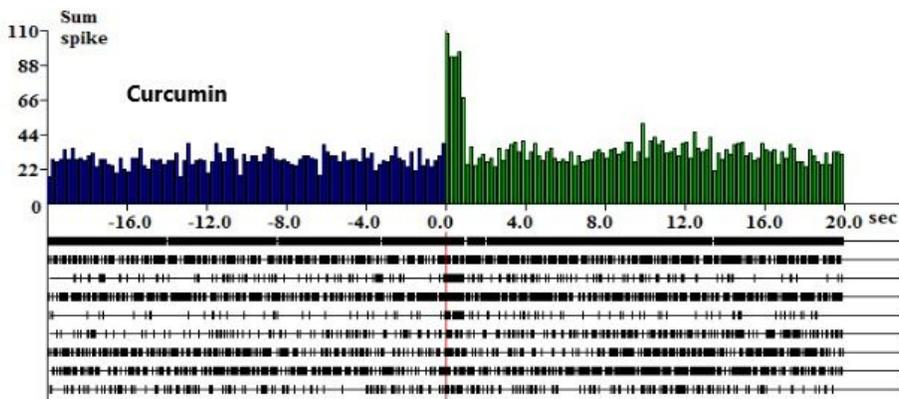
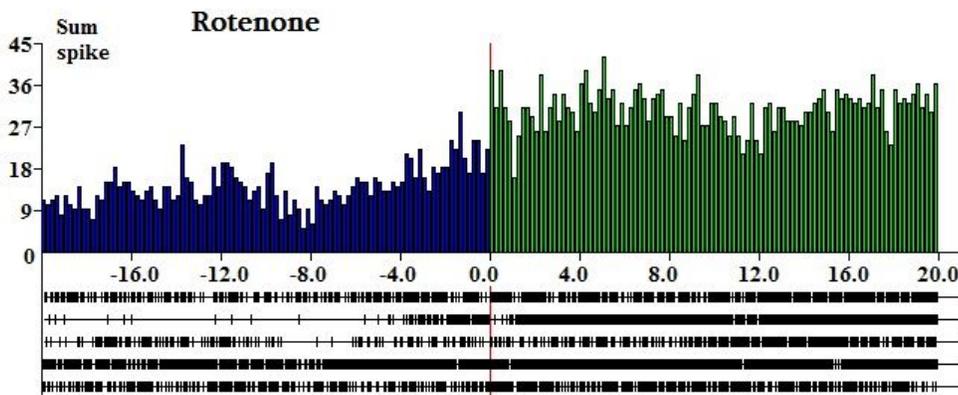
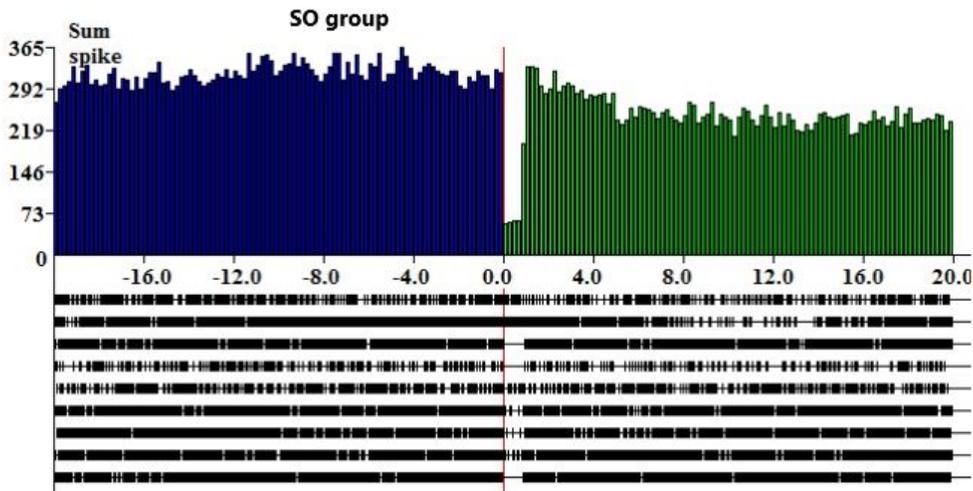


Figure 1

Effects of Curcumin in the rotenone-induced PD rats. Curcumin+R+S effects on SNc neuronal activity in response to HFS (100 Hz) of hippocampus was evaluated electrophysiologically.

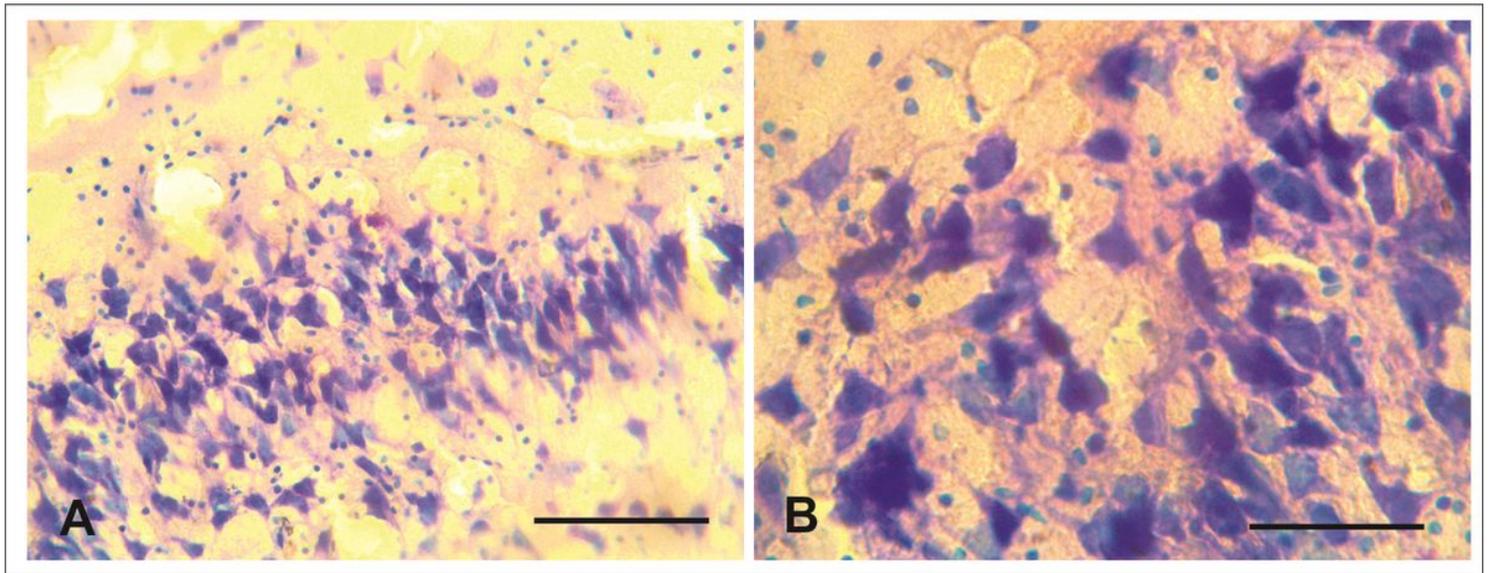


Figure 2

Hippocampal neurons in rotenone-induced PD rats: A-rotenone, B- Curcumin. Significant morphological changes in cell area or shape were shown by Nissl staining. The neuronal degeneration was shown by distorted neuronal cells, shrinkage of nuclei, dark staining in the hippocampus of rotenone treated rats. Photomicrographs ($\times 100$) of cresyl violet acid staining in brain hippocampus CA1 sections (in slices of $5 \mu\text{m}$).

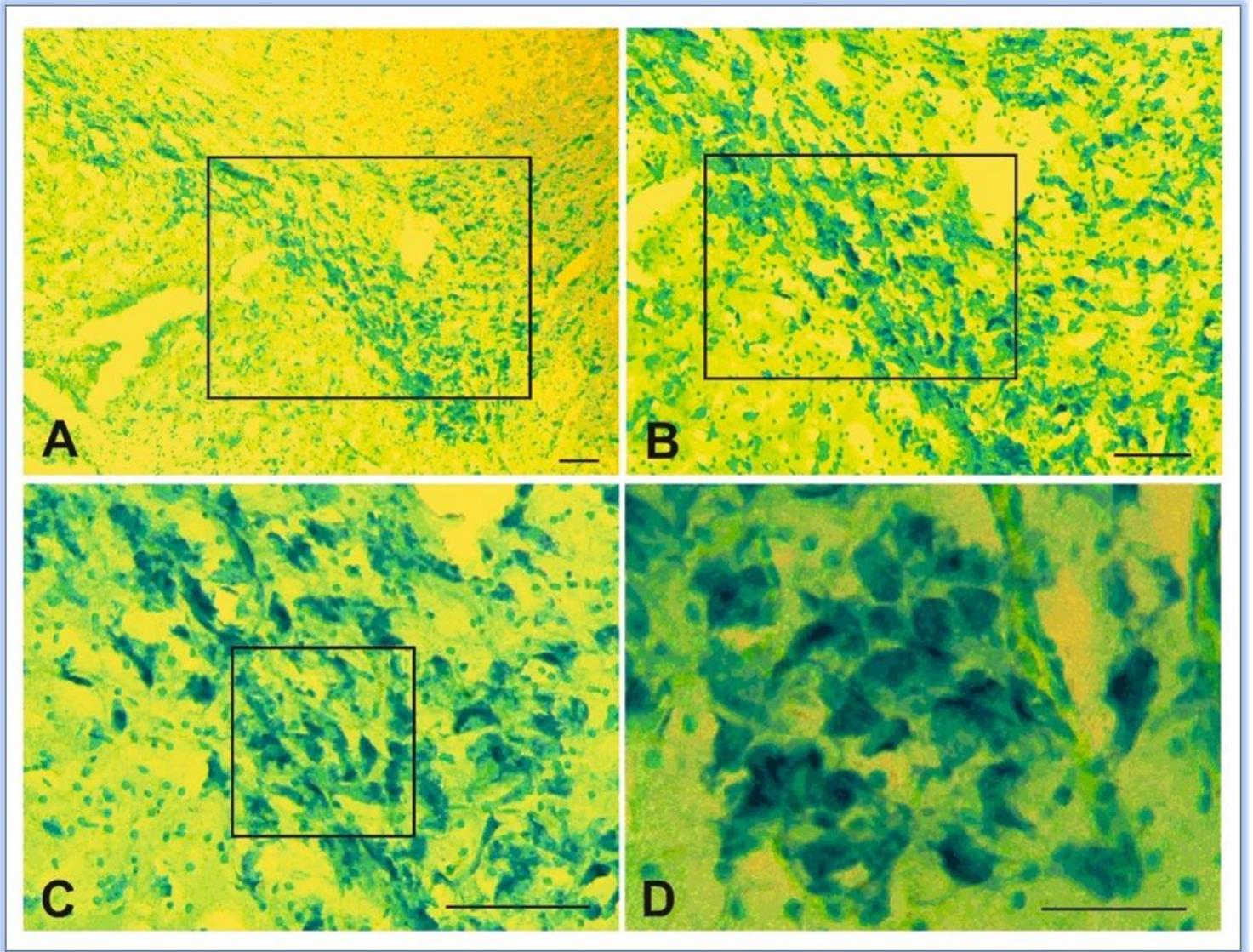


Figure 3

Representative Nissl-staining (methylene green) of nigral neurons from brain sections (SNc-substantia nigra pars compacta) corresponding to all animal groups. A: Rotenone group; B: SO-treated control group; C and D Curcumin-treated animals. Scale $\bar{\square}$ A-C 100 μ M, D-50 μ M