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Rotenone-Induced Dysregulation of Synaptosomal Ca^{2+} And Hypokinetic Behavior in A Rat Model of Parkinsonism

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Abstract

Background: Rotenone, a lipophilic mitochondrial complex I inhibitor, is widely used to model Parkinsonian neurodegeneration and synaptic failure.

Objective: To quantify rotenone-associated behavioral changes and determine whether they co-occur with altered glutamate-evoked synaptosomal Ca^{2+} dynamics.

Methods: Adult male Wistar rats were randomized into control and rotenone groups (2.5 mg/kg/day, i.p., 11 consecutive days; n=6/group). Exploratory behavior was assessed in an open-field/hole-board ar ena (42 × 42 cm; 42-floor grid; 3 min; ~100 lux). Crude synaptosomes (P2 fraction) were isolated from whole brain tissue, loaded with Fluo-4AM (final 5 µM, 30 min, 37°C), and stimulated with L-glutamate (50 µM). Ca^{2+} responses were summarized as resting signal, peak amplitude, area under the curve (AUC), and clearance time constant (τ).

Results: Rotenone reduced horizontal locomotion (72±6 vs 14±4 crossings), vertical activity (38±5 vs 8±3 rearings), and hole-poking (16±2 vs 6±1; all $p<0.05$). Synaptosomes from rotenone-treated rats displayed a higher resting Ca^{2+}-related

fluorescence (+21%) and enhanced glutamate-evoked Ca^{2+} responses (peak +18%, AUC +25%), alongside faster decay (τ −17%) relative to controls ($p<0.05$).

Conclusions: Subchronic rotenone exposure produces a reproducible hypokinetic phenotype that parallels presynaptic Ca^{2+} dysregulation, supporting a mechanistic link between mitochondrial stress and abnormal glutamate-triggered Ca^{2+} signaling.

Keywords: Parkinsonism; rotenone; synaptosomes; Ca^{2+} homeostasis; Fluo-4AM; glutamate; oxidative stress.

Abbreviations: AUC, area under the curve; EGTA, ethylene glycol tetraacetic acid; NCX, Na^{+}/Ca^{2+} exchanger; PMCA, plasma membrane Ca^{2+}-ATPase; ROS, reactive oxygen species.

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1. Introduction

Parkinson's disease (PD) is characterized by progressive dopaminergic dysfunction and widespread synaptic alterations that can precede overt neuronal loss. Experimental PD models that target mitochondrial bioenergetics are valuable because mitochondrial impairment and oxidative stress are increasingly recognized as upstream drivers of synaptic failure. Rotenone, a lipophilic inhibitor of mitochondrial complex I, reproduces key motor features of PD in rodents and is associated with elevated ROS and impaired ATP production [1,2,8].

At the synapse, Ca^{2+} signals operate as a biophysical "control variable" that gates neurotransmitter release, vesicle recycling, and forms of short- and long-term plasticity. Even modest shifts in resting Ca^{2+} levels, response amplitudes, or clearance kinetics can change synaptic gain and promote maladaptive activation of Ca^{2+}-dependent enzymes. During mitochondrial stress, Ca^{2+} buffering and extrusion mechanisms (mitochondrial uptake, PMCA, and NCX) become energetically constrained and redox-sensitive, providing a plausible route from complex I inhibition to altered presynaptic Ca^{2+} dynamics [4,5,7].

Glutamate-triggered Ca^{2+} influx is particularly relevant because excessive or poorly controlled Ca^{2+} entry can amplify oxidative injury and accelerate synaptic rundown. Although postsynaptic NMDA receptor overactivation is a well-known excitotoxic pathway, presynaptic glutamate receptors and voltage-gated Ca^{2+} channels can also contribute to Ca^{2+} loading and transmitter imbalance. Therefore, synaptosome-based Ca^{2+} readouts provide a reductionist but mechanistically informative window into presynaptic Ca^{2+} handling under pathological conditions [16-20].

The present study tested whether subchronic rotenone exposure induces (i) measurable hypokinesia in open-field and balance-beam tasks and (ii) parallel alterations in glutamate-evoked Ca^{2+} signals in rat brain synaptosomes. We hypothesized that rotenone would increase resting Ca^{2+}-related fluorescence and potentiate glutamate-evoked responses while perturbing clearance kinetics, consistent with mitochondrial redox–bioenergetic constraints on Ca^{2+} homeostasis.

2. Methods

2.1. Ethics statement. All procedures were approved by the Institutional Animal Care and Use Committee of Institute of Biophysics and Biochemistry, National University of Uzbekistan (protocol No. 4, 17 February 2026) and complied with national guidelines for laboratory animal welfare.

2.2. Animals and experimental design. Adult male Wistar rats (220–250 g) were housed under standard conditions (12 h light/dark cycle; food and water ad libitum).

Animals were randomly allocated to two groups (n=6/group): Control and Rotenone. Behavioral scoring and Ca^{2+} analysis were performed blinded to group assignment.

2.3. Rotenone-induced Parkinsonism model. Rotenone was administered intraperitoneally at 2.5 mg/kg/day for 11 consecutive days; controls received vehicle i.p. (vehicle composition: Rotenone (50 mg) was weighted in standard weighing machine and dissolved in 1 mL 100% di methyl sulfoxide (DMSO) (Sigma-Aldrich Chemicals, USA). About 0.1 mL of DMSO mixture was added to 9.9 mL olive oil to get a solution of 10 mL, with the final concentration of rotenone 0.5 mg/mL. 8 Before starting any new experiment begin, fresh stock solution of rotenone was prepared, protected from light). The dose and schedule were selected to elicit reproducible motor slowing in rats [1,2].

2.4. Open-field/hole-board testing. Exploratory activity was evaluated in a square arena (42 × 42 cm; opaque walls; floor divided into 42 equal squares) equipped with holes for nose-poking. Illumination was maintained at ~100 lux. Animals were recorded for 3 min and the following endpoints were quantified: horizontal locomotion (square crossings), vertical activity (rearings), grooming episodes, hole-poking, and fecal boluses. The arena was cleaned between animals to reduce olfactory cues. This task is commonly used to capture hypokinesia and anxiety-like components in rotenone-based PD models [10].

2.5. Balance beam task. Motor coordination was assessed using a 1-m beam with a safety box at the end. Animals were trained for two familiarization trials prior to testing. During testing, latency to cross, number of stops, and falls were recorded [6].

2.6. Synaptosome preparation. Whole brains were rapidly removed on ice, homogenized in ice-cold 0.32 M sucrose buffer (pH 7.4) containing protease inhibitors, and processed to obtain the crude synaptosomal pellet (P2) [15]. Homogenates were centrifuged at 1,000×g for 10 min (4°C) to remove nuclei and debris; the supernatant was centrifuged at 12,000×g for 15 min to obtain P2. The pellet was resuspended in Krebs–HEPES buffer (in mM: NaCl 140, KCl 5, $MgCl_2$ 1, glucose 10, HEPES 10; pH 7.4). Protein concentration was determined using the Lowry method with bovine serum albumin as standard.

2.7. Fluo-4AM loading and Ca^{2+} measurements. Synaptosome suspensions were loaded with Fluo-4AM (final 5 μM; stock prepared in anhydrous DMSO, protected from light) for 30 min at 37°C with gentle mixing, then washed and resuspended in recording buffer. Fluorescence was recorded at 37°C using a fluorescence reader/fluorometer (USB-2000, USB2E7916. OceanOptics.USA) with excitation/emission set to 494/516 nm. Signals were normalized to baseline (F/F_0). After a 60-s baseline, glutamate (50 μM) was applied and fluorescence was recorded for 180 s. EGTA (1 mM) was used in selected experiments to reduce extracellular free Ca^{2+} [11-14].

2.8. Quantification of Ca^{2+} responses. Resting signal was defined as the mean F/F_0 during the 10-s pre-stimulus window. Peak amplitude was the maximal F/F_0 within 30 s after stimulation. AUC was calculated by trapezoidal integration over 0–180 s. The decay time constant (τ) was estimated by fitting the post-peak segment to a mono-exponential decay.

2.9. Statistical analysis. Data are presented as mean ± SEM. Normality was assessed using the Shapiro–Wilk test. Between-group comparisons used two-tailed Student's t-test (single outcomes) or two-way ANOVA (factors: model × condition) with post-hoc tests where appropriate. If assumptions were violated, non-parametric alternatives were applied. A p-value <0.05 was considered significant.

3. Results

3.1. Behavioral phenotype. Rotenone-treated rats exhibited a marked reduction in exploratory locomotion and novelty-seeking behaviors in the open-field/hole-board test (Table 1). Horizontal crossings decreased by ~81% and vertical activity (rearings) by ~79% ($p<0.05$), consistent with a hypokinetic phenotype. Reductions in grooming and hole-poking further suggested diminished exploration.

Balance-beam performance was also impaired in the rotenone group, with increased traversal latency and more frequent stops and falls, consistent with compromised motor coordination.

3.2. Synaptosomal Ca^{2+} dynamics. Baseline synaptosomal fluorescence [24-27] was higher in the rotenone group than in controls (≈+21%; Table 2), consistent with elevated resting Ca^{2+} load or reduced buffering/extrusion capacity. Upon L-glutamate

stimulation (50 μM), synaptosomes from rotenone-treated rats showed a larger peak response (+18%) and greater integrated Ca^{2+} load (+25% AUC) relative to controls ($p<0.05$). The decay constant τ was shorter (≈−17%), indicating an altered response time-course under rotenone condition.

Overall, rotenone exposure reshaped both amplitude and kinetics of glutamate-triggered synaptosomal Ca^{2+} signals, paralleling the behavioral hypokinesia.

Table 1. Open-field/hole-board indicators in control and rotenone groups.

Values are mean ± SEM (n=6/group). *p<0.05 vs control.

Group	Vertical activity (rearing, n)	Horizontal locomotion (crossings, n)	Grooming (episodes, n)	Hole-poking (n)	Fecal boluses (n)
Control	38±5	72±6	23±3	16±2	5±1
Rotenone	8±3*	14±4*	9±2*	6±1*	2±1*

Table 2. Glutamate-evoked synaptosomal Ca^{2+} response metrics (normalized).

Control group mean is set to 1.00. Values represent normalized mean effect sizes; p-values are derived from statistical testing of the underlying raw metrics (n=6/group).

Metric	Control (normalized)	Rotenone (normalized)	Effect (Δ%)	p-value
Resting signal	1.00	1.21	+21%	<0.05
Peak amplitude	1.00	1.18	+18%	<0.05
AUC (0–180 s)	1.00	1.25	+25%	<0.05
τ (decay)	1.00	0.83	−17%	<0.05

4. Discussion

This study links a subchronic rotenone regimen that produces clear hypokinesia in rats to a measurable distortion of presynaptic Ca^{2+} dynamics in crude brain synaptosomes. The key observation is a coordinated shift in Ca^{2+} "set-points" (higher resting signal) and "gain" (larger glutamate-evoked peak and AUC), alongside altered decay kinetics.

Rotenone impairs electron transport at complex I, decreasing ATP availability and increasing ROS generation [1,2,8]. In presynaptic terminals, ATP shortage constrains active Ca^{2+} extrusion (PMCA) and Na^+/K^+-ATPase function, indirectly altering NCX driving force. In parallel, oxidative modification of membrane lipids and proteins can change channel gating and transporter kinetics. Together, these processes predict elevated resting Ca^{2+} and larger Ca^{2+} transients for a given stimulus, consistent with our synaptosomal results [4,5,7].

In our assay, L-glutamate was used as the stimulus; therefore, the measured Ca^{2+} responses should be interpreted as a composite readout reflecting ionotropic and/or metabotropic glutamate receptors together with depolarization-driven voltage-gated Ca^{2+} entry. Because selective receptor/channel pharmacology (e.g., MK-801/APV, CNQX, ω-conotoxin) was not applied, receptor subtype attribution remains cautious. Nevertheless, the direction of change-enhanced amplitude and AUC-fits an excitotoxic "set-point shift" model in which glutamatergic stimulation produces disproportionate Ca^{2+} accumulation under mitochondrial stress [4,5,21-23].

The shorter decay constant τ observed under rotenone condition suggests that rotenone affects not only Ca^{2+} entry but also the kinetics of signal termination. This could reflect compensatory acceleration of extrusion, faster inactivation of Ca^{2+} entry pathways, or response truncation due to synaptosomal stress. Accordingly, τ should be treated as a kinetic signature motivating targeted dissection of entry versus clearance mechanisms.

Finally, behavioral hypoactivity in open-field and balance-beam tasks is compatible with basal ganglia circuit dysfunction. Presynaptic Ca^{2+} dysregulation can contribute by altering release probability, promoting vesicle pool depletion, and amplifying oxidative injury through Ca^{2+}-dependent enzyme activation. Thus, synaptosomal Ca^{2+} metrics may serve as tractable biophysical markers linking mitochondrial injury to behavioral phenotype.

Limitations include the use of crude synaptosomes from whole brain (regional heterogeneity) and the absence of selective pharmacological dissection and mitochondrial readouts. Future work should incorporate region-specific fractions, receptor/channel panels, and direct oxidative/mitochondrial measures to strengthen causal inference.

5. Conclusions

Subchronic rotenone treatment (2.5 mg/kg/day, i.p., 11 days) induced marked hypokinesia and was accompanied by presynaptic Ca^{2+} dysregulation in brain synaptosomes. Rotenone increased resting Ca^{2+}-related fluorescence and amplified glutamate-evoked Ca^{2+} responses, supporting a mechanistic link between mitochondrial stress and abnormal Ca^{2+} signaling. Synaptosomal Ca^{2+} dynamics therefore provide a practical biophysical readout for Parkinsonian mitochondrial injury and a platform for testing candidate neuroprotective interventions.

Declarations

Conflicts of Interest: The authors declare no competing interests.

Data Availability: Data are available from the corresponding author upon reasonable request.

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