Oxidative stress in vagal neurons promotes Parkinsonian pathology and intercellular α-synuclein transfer

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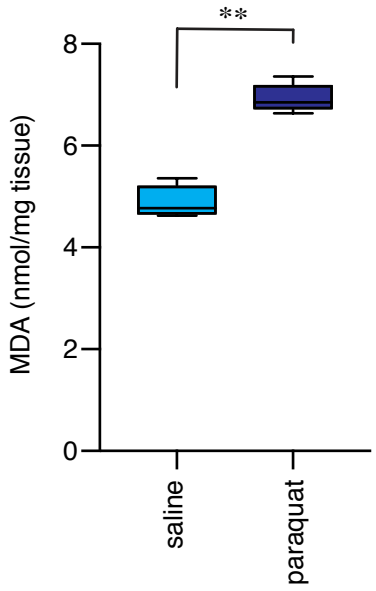
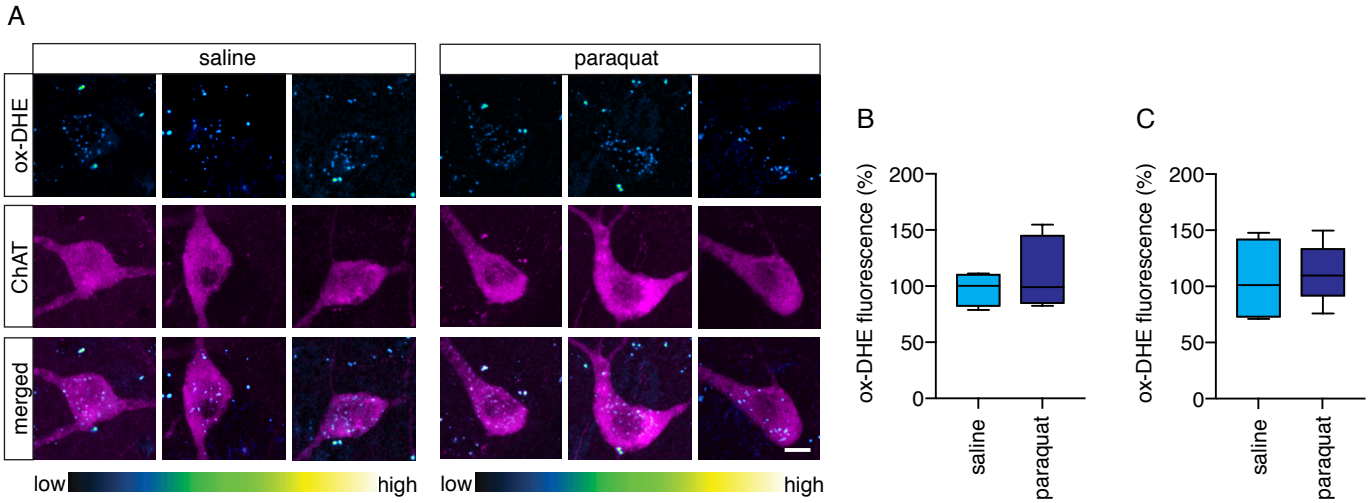
Supplemental Material

Supplemental Figure 1



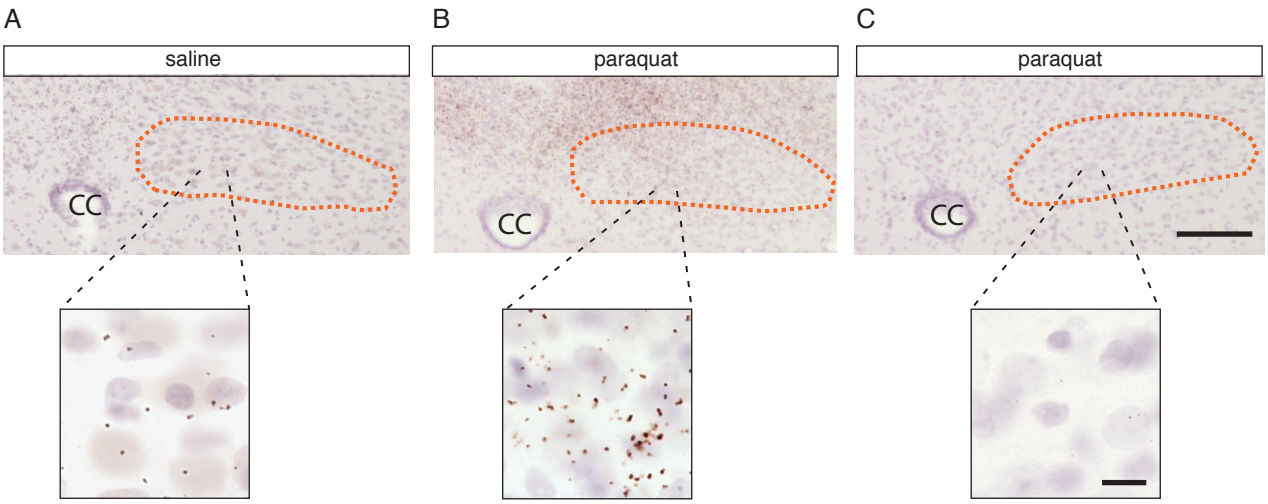
Supplemental Figure 1. Paraquat exposure causes an increase in lipid peroxidation in the DMnX. Mice received 2 intraperitoneal injections of either saline (n=5) or paraquat (n=5) separated by a 1-week interval and were sacrificed at 2 days post treatment. DMnX-containing specimens of the dorsal medulla oblongata were used for measurements of malondialdehyde (MDA) using a colorimetric assay. Box and whisker plots show median (middle line), upper and lower quartiles, and the maximum and minimum as whiskers. $^{**}P \leq 0.01$, Mann-Whitney test.

Supplemental Figure 2



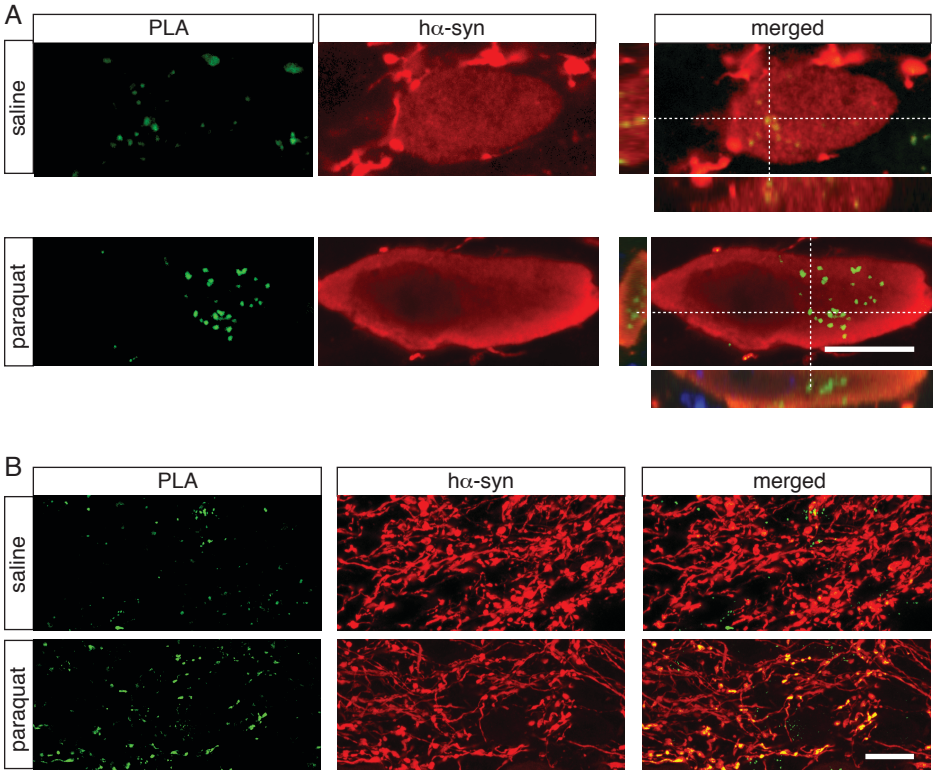
Supplemental Figure 2. Paraquat administration does not cause overt oxidative stress within cholinergic neurons in the striatum and medial septal nucleus. Mice received 2 intraperitoneal injections of either saline or paraquat separated by a 1-week interval and were sacrificed at 2 days post treatment. They were also injected with DHE 1 hour before the time of sacrifice. (**A**) Representative confocal images show ox-DHE fluorescence (blue-green-yellow color graded) within neurons immunoreactive for choline acetyltransferase (ChAT, magenta) in the striatum. Three neurons from 3 different animals injected with saline or paraquat are shown. Scale bar: 5 μm. (**B** and **C**) Comparison of the integrated density of fluorescent ox-DHE puncta within cholinergic (ChAT-positive) neurons in the striatum (B) and medial septal nucleus (C) from mice treated with saline (n=4, azure bar) or paraquat (n=5, dark blue bar). Approximately 14 and 12 neurons/animal were analyzed and averaged in the striatum and medial septal nucleus, respectively. Values were calculated as percent of the mean value in saline-injected animals. Box and whisker plots show median, upper and lower quartiles, and the maximum and minimum as whiskers.

Supplemental Figure 3

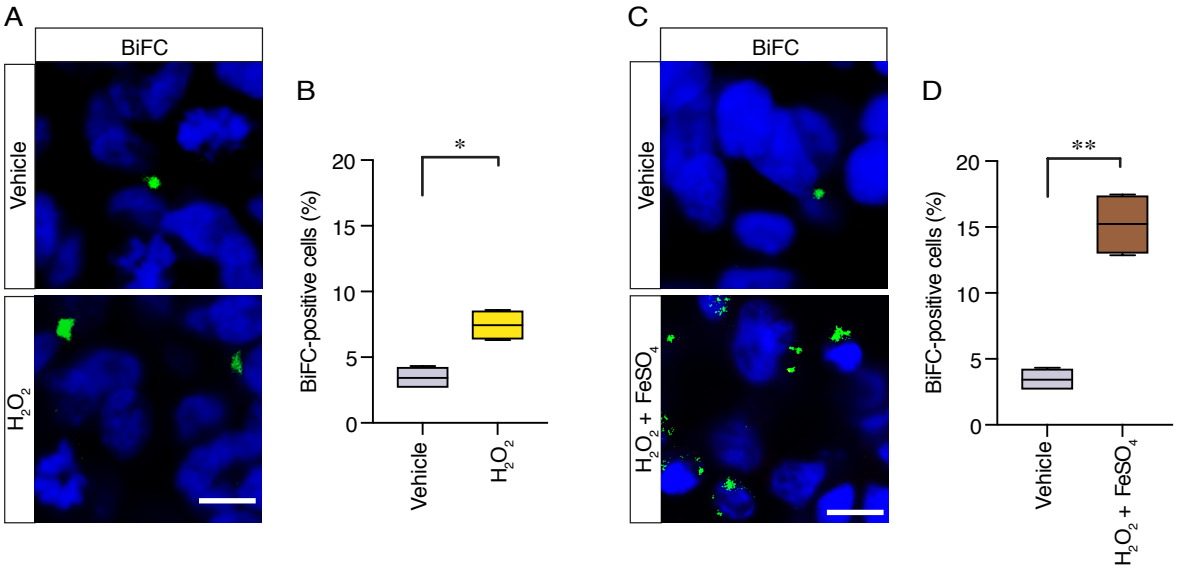


Supplemental Figure 3. Oxidative stress causes nitration of hα-synuclein that can be detected by specific PLA-generated signal. Mice received an injection of hα-synuclein-carrying AAVs into the left vagus nerve, were treated with either saline or paraquat and sacrificed at 2 days post treatment. (**A** and **B**) Representative low-(top panels) and high-(bottom panels) magnification images of the left DMnX (delineated in orange at low magnification) show specific signal for nitrated hα-synuclein detected by direct hα-synuclein/3-NT PLA. (**C**) Tissue sections of the medulla oblongata from a paraquat-injected mouse were pre-incubated with a cocktail of antibodies against nitrated α-synuclein and then processed for direct hα-synuclein/3-NT PLA. Representative images show that, in these tissue specimens, the PLA signal was abolished. CC=central canal. Scale bars: 100 µm (low) and 10 µm (high magnification).

Supplemental Figure 4



Supplemental Figure 4. Oxidative stress causes intraneuronal accumulation of oligomeric hα-synuclein. Mice received an injection of hα-synuclein-carrying AAVs into the left vagus nerve, were treated with either saline or paraquat and sacrificed at 2 days post treatment. Sections of the medulla oblongata were double-labeled with syn/syn PLA and anti-hα-synuclein. (**A**) Representative images show neuronal cell bodies in the left DMnX. Co-localization was confirmed in merged images showing orthogonal cross-sections in the x–z and y–z axes. Scale bar: 5 µm. (**B**) Representative images show neuritic labeling in the left DMnX. Syn/syn PLA and hα-synuclein co-localization (merged panels) was enhanced in the paraquat-treated animal. Scale bar: 10 µm.



Supplemental Figure 5. ROS-induced oxidative stress promotes cell-to-cell hα-synuclein transfer in vitro. Co-cultures of V1S- and SV2-expressing cells were incubated for 2 days with vehicle, 100 µM hydrogen peroxide or 100 µM hydrogen peroxide plus 10 mM iron sulfate. At the end of these incubations, no significant differences in cell viability were found among the 3 treatment groups (data not shown). (**A** and **C**) Representative images show BiFC (green) as a marker of hα-synuclein transfer into recipient cells. Scale bar: 10 µm. (**B** and **D**) The percent of BiFC-positive cells (n=4 separate experiments/treatment) was compared in cultures treated with vehicle vs. hydrogen peroxide (B) or with vehicle vs. hydrogen peroxide plus iron sulfate (D). For each experiment, at least 1000 cells were analyzed and values were averaged. Box and whisker plots show median (middle line), upper and lower quartiles, and the maximum and minimum as whiskers. *$P \leq 0.05$, **$P \leq 0.01$, Mann-Whitney test.

Supplemental Table 1

Primary antibodies used in the study

Protein target	Antibody	Host	Supplier/ Catalog	Application	Concentration
α-Synuclein (human-specific)	Monoclonal clone MJFR1	Rabbit	Abcam ab138501	IF BM PLA (direct) PLA (indirect)	1:20,000 1:20,000 1 mg/ml 1:5,000
α-Synuclein (human-specific)	Monoclonal clone Syn 211	Mouse	Abcam 36-008	BM PLA (direct)	1:30,000 1 mg/ml
α-Synuclein (human-specific)	Monoclonal clone 4B12	Mouse	BioLegend 103-108	IF	1:500
α-Synuclein (human-specific)	Monoclonal clone 15G7	Rat	Enzo ALX-804-258	IF	1:1,000
α-Synuclein (total)	Monoclonal clone Syn-1 42/α-synuclein	Mouse	BD Biosciences 610787	ELISA	1 µg/ml
Choline acetyltransferase	Polyclonal	Goat	Millipore AB144P	IF	1:200
Nitrated α-synuclein	Monoclonal clone nSyn24.8	Mouse	Thermo Fisher MA5-16142	IF BM PLA blocking	1:100 1:500 1:50
Nitrated α-synuclein	Monoclonal clone nSyn12	Mouse	Upstate 36-011	PLA blocking *In vitro* blocking	1:20 50 µg/ml
Nitrated α-synuclein	Monoclonal clone nSyn14	Mouse	Upstate 36-012	PLA blocking	1:20
3-Nitrotyrosine (3-NT)	Monoclonal clone 39B6	Mouse	Abcam ab61392	IF *In vitro* blocking PLA (direct) PLA (indirect) ELISA	1:400 50 µg/ml 1 mg/ml 1:400 1 µg/ml
Oxidized α-synuclein	Monoclonal clone Syn 505	Mouse	Thermo Fisher 35-8300	IF	1:1,000
Aggregated α-synuclein	Monoclonal clone Syn-O2	Mouse	Custom	IF	1:5,000
Fibrillar α-synuclein	Monoclonal clone Syn-F1	Mouse	Custom	IF	1:5,000

IF = immunofluorescence; BM = brightfield microscopy; PLA = proximity ligase assay;
ELISA = Enzyme-linked immunosorbent assay
Custom antibodies were a kind gift from Dr. Omar El-Agnaf