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Original Article

Low doses of single or combined agrichemicals induces α-synuclein aggregation in nigrostriatal system of mice through inhibition of proteasomal and autophagic pathways

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Abstract: Alpha synuclein (SNCA) genes and environmental factors are important risk factors of Parkinson's disease (PD). The agrichemicals paraquat, maneb and chlorpyrifos selectively target dopaminergic neurons, leading to parkinsonism, through ill-defined mechanisms. We analyzed the ability of low dose paraquat, maneb and chlorpyrifos, separately or combined together, to induce synucleinopathy in wild type mice. Paraquat and maneb applied together did not increase α-Synuclein (α-Syn) levels. By contrast, paraquat and chlorpyrifos together resulted in robust accumulation of α-Syn in striata in mice. Therefore, co-treatment with chlorpyrifos enhanced the effects of paraquat. Paraquat, and its co-treatment with maneb or chlorpyrifos, inhibited all soluble proteasomal expression of 26S proteasome subunits. Both paraquat and chlorpyrifos treatments increased levels of the autophagy inhibitor, mammalian target of rapamycin, mTOR, suggesting impaired axonal autophagy, despite increases in certain autophagic proteins, such as beclin 1 and Atg 12. Autophagic flux was also impaired, as ratios of LC3 II to LC3 I were reduced in all the treated animals. These results suggest that a combination of paraquat and chlorpyrifos is much more toxic than paraquat alone or combined with maneb. These effects are likely *via* inhibitory effects of these toxins on proteasomes and autophagy, which lead to accumulation of α-Syn. Our study provides a novel insight into the mechanisms of action of these agrichemicals.

Keywords: Alpha synuclein, Parkinson's disease, paraquat, maneb, chlorpyrifos, ubiquitin-proteasome system

Introduction

Parkinson's disease (PD) results from dopaminergic cell body depletion in the nigrostriatal system, and is one of the most common neurodegenerative diseases-only second to Alzheimer's disease (AD). PD has been typically diagnosed by the presence of Lewy bodies, which are primarily composed of α-Syn protein aggregates in DA neurons. However, the molecular mechanism that triggers the disease is still under debate [1], even though impaired protein degradation is suggested in the aggregation of α-Syn, and the ubiquitin-proteasome system (UPS) and autophagy-lysosomal pathway (ALP) are involved in intracellular degradation processes for impaired protein α-Syn.

Epidemiological studies have suggested that agrichemicals and factors linked to pesticide exposure may play a causal role in PD pathogenesis [2-5]. The herbicide paraquat (PQ), a substance structurally similar to the neurotoxin precursor MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), has emerged as a putative neurotoxicant that adversely impacts dopamine (DA) systems [6, 7]. A growing body of evidence supports accumulation of α-synuclein (α-Syn) within nigrostriatal neurons in PQ treated mice [8]. In addition, low concentrations of PQ can induce several characteristics of autophagy in human neuroblastoma SH-SY5Y cells and reduce proteasomal function in DJ-1 (a cancer- and PD-associated protein, associated with autosomal recessive early onset parkinsonism) deficient mice [9, 10]. Besides MPTP and PQ, studies have shown that the fungicide maneb (MB), and the insecticide chlorpyrifos (CPF), may also contribute to decreasing locomotor

activity and affecting DA neurotransmission [11-13]. MB has been shown to inhibit the proteasomal activity in cultured cells and *in vivo*, thus impairing clearance of dysfunctional proteins [14, 15]. The *in vivo* effects of CPF on proteasomes and autophagy are still not known. The agrichemicals PQ, MB and CPF all can be absorbed by the gastrointestinal tract, skin and respiratory tract [16, 17].

Co-exposures of these agrichemicals could cause additive or synergistic toxicity, as indicated in some PD studies [4, 18, 19]. Humans are regularly exposed to agrichemical mixtures, as evidenced by the detection of multiple agrichemical residues in food or tobacco [20, 21]. Several studies proposed that combined PQ and MB exposures would produce greater effects on DA systems than would either compound administered alone [8-11, 18]. Some other studies indicated that PQ is much more toxic than PQ+MB [15]. In the current study, we set out to analyze the ability of low doses of paraquat, maneb and chlorpyrifos, separately or together, to induce synucleinopathy in wild type mice.

Materials and methods

Chemicals and antibodies

The antibodies used in this study are: anti-α-Syn (1:1000), anti-β-actin (1:1000) from Santa Cruz Biotechnology (Dallas, USA); anti-Proteasome 19S Subunit S4 (1:1000) and anti-Proteasome 20S Subunit α5 (1:1500) from Abcam (Cambridge, UK); anti-Atg 12 (D88H11) (1:1000), anti-LC3B (1:1500), anti-mTOR (1:1000), anti-Beclin 1 (1:1000) from Proteintech Group (Chicago, USA). The agrichemicals used in this study are: Paraquat (10 mg/kg), Maneb (30 mg/kg), Chlorpyrifos (3 mg/kg) from Sigma (St. Louis, USA). All other chemicals, if not specified, were at least analytical grade and were purchased from Sigma (St. Louis, USA).

Animals

Mice used in these studies were 8 weeks old males with a mixed C57BL/6NCrlVr background. All studies with animals were conducted under the strict guidelines of the National Institutes of Health of China and were approved by China Medical University Animal Care and Use Committee.

Treatment of mice with PQ, PQ and MB, PQ and CPF

Male mice on a mixed C57BL/6NCrlVr background received intraperitoneal injections of 10 mg/kg PQ or 10 mg/kg PQ combined with 30 mg/kg MB (PQ+MB) in sterile filtered 0.9% saline or 10 mg/kg PQ combined with 3 mg/kg CPF (PQ+CPF) in coil oil respectively, for the rat acute oral LD50 of PQ is up to 150 mg/kg, MB up to 7500 mg/kg and CPF up to 163 mg/kg (according to the instructions). Control mice were injected with saline and coil oil (vehicle treated). Since PQ+MB, PQ+CPF were administered as separate injections, two separate injections of saline and coil oil were administered to control mice, PQ and saline injections were administered to the PQ group, so that all animals received the same number of injections and the same total volume of injected saline or coil oil (adjusted for weight). Treatment was administered twice weekly for 4 weeks (8 total injections) then all animals were sacrificed. Striata were dissected for immunocytochemistry and Western blot analysis.

Immunocytochemistry

Brains were fixed in 4% paraformaldehyde, and for single immune-labeling studies, 4 μm sections were incubated in primary antibody for 24 h at 4°C, followed by 1 h incubation with biotinylated secondary antibody at room temperature. The avidin-biotin complex method [22] was used to detect the antigen signal and 3,3'-diaminobenzidine tetrachloride (DAB) was used to visualize the final product. The primary antibodies used were monoclonal mouse antibody against tyrosine hydroxylase (TH) (1:200). Secondary antibodies used were biotinylated goat anti-mouse immunoglobulin G (IgG) or biotinylated goat anti-rabbit IgG (1:200). For controls, one or both primary antibodies were omitted. We examined immunostained sections using bright-field microscopy. Images were captured on a Leitz microscope (Leica, Wetzlar, Germany) linked to image analysis system (MPIAS-500) with selective filter sets. For final output, images were processed using Adobe Photoshop 5.0 software (Adobe, San Jose, USA).

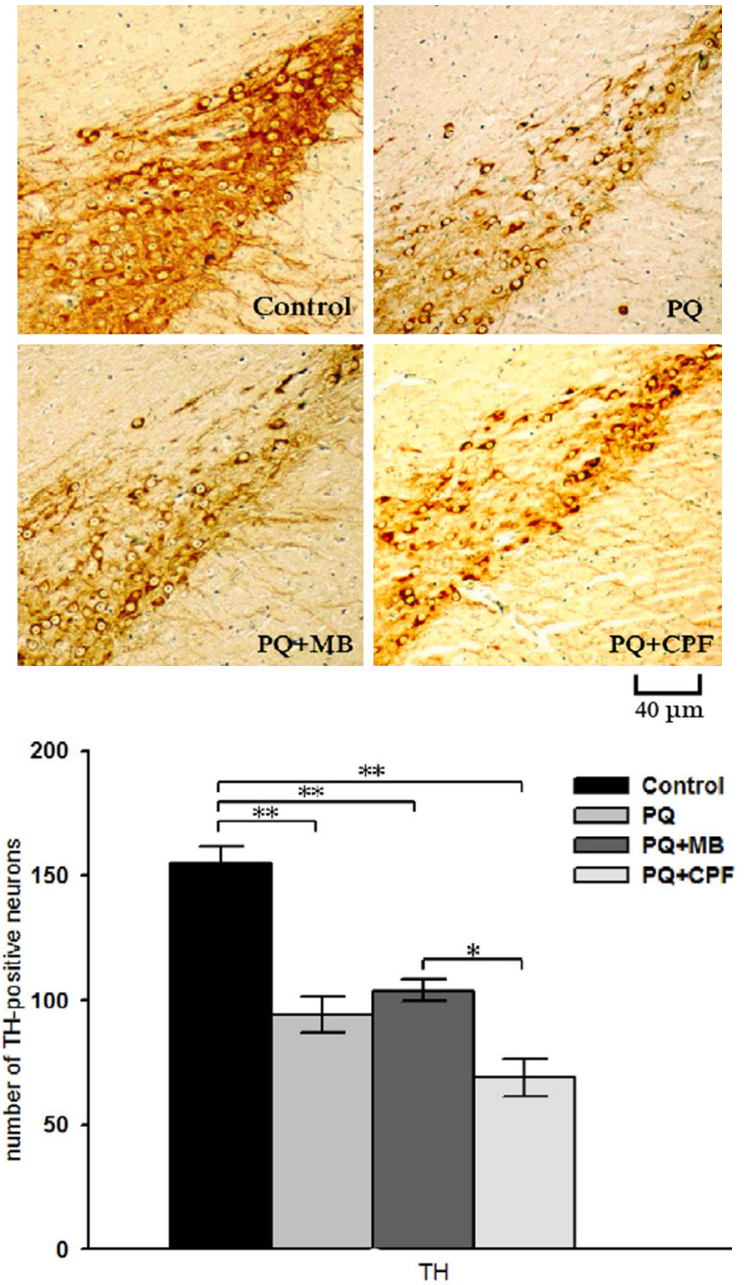

Figure 1. Tyrosine hydroxylase (TH)-immunoreactivity in striata. Mice were treated with four different injections of control, paraqua (PQ) (10 mg/kg), paraqua (PQ) + maneb (MB) (10 mg/kg + 30 mg/kg) and paraqua (PQ) + chlorpyrifos (CPF) (10 mg/kg + 3 mg/kg) twice weekly. TH-immunohistochemistry was performed at 48 h after the last injection. A constant total number of TH^+ neurons in striata of each treated mice was observed. The bar graphs are composites of TH^+ neurons numbers summarized from all animals (n = 5-6). Values are mean ± SEM as compared to the control mice. Asterisks (*, **) indicate values significantly different from each group ($P<0.05$, $P<0.01$). Student's t-test was performed to compare control and treatment groups for all data.

Preparation of lysates for western blots

Tissues were homogenized in buffer containing 10 mM Tris-HCl, pH 7.4, 1 M NaCl, 250 mM sucrose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 10 mg/ml each of phosphatase inhibitor cocktail and protease inhibitor cocktail tablets. Sodium cholate (20% in water, wt/vol) was added to a final concentration of 1% (vol/vol). The mixture was left on ice for 30 min, with brief vortexing every 5 min, followed by centrifugation for 20 min at 150000× g at 4°C. The clear supernatant, representing the cholate soluble fraction, was collected. Samples were diluted to equal protein concentrations with homogenizing buffer, then further diluted to 1:4 with dilution buffer (50 mM Tris-HCl, pH 7.4, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA), so that final sodium cholate concentration was 0.2%. Samples were then mixed with Laemmli buffer (1:1 vol/vol) and analyzed by Western blotting.

Western blot analysis

Samples were analyzed on 10-20% Tris-HCl Criterion gels and transferred to a PVDF (polyvinylidene difluoride) membrane (Merck Millipore, Billerica, USA). The blots were then blocked with 20 mM Tris-buffered saline, pH 7.6 containing 0.1% Tween 20 (TBST) and 5% (wt/vol) blotting grade blocker non-fat dry milk for 1 hour at room temperature. Western blots were probed with an array of primary antibodies diluted in TBST with 5% milk at experimentally determined concentrations ranging from 1:1000 to 1:1500. After incubation for 2 hours at room temperature with HRP-conjugated secondary antibodies (1:3000 to 1:5000), proteins were revealed by enhanced chemiluminescence (Perkin Elmer). Images were scanned by Scanner EPSON Perfection V700 Photo (Seiko Epson, Nagano Prefecture,

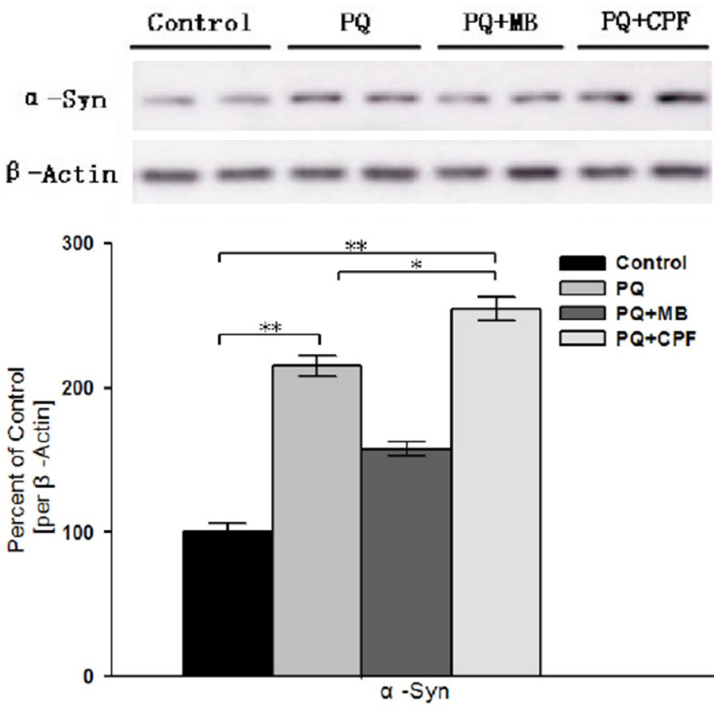

Figure 2. Effects of paraqua (PQ), maneb (MB), and/or chlorpyrifos (CPF) on α-Synuclein in striata. Striata from control, PQ, PQ+MB and PQ+CPF injected mice were dissected and homogenized in a modified RIPA buffer containing 1.0% sodium cholate. Cholate-soluble fractions were isolated by centrifugation and analyzed by Western blots, as described under "Materials and Methods". After exposure to antibodies, blots were stripped and re-probed for other antibodies The blots show representative gels while the bar graphs are composites of blots summarized from all animals (n = 5-6). α-Syn were expressed relative to β-actin used as a loading control. Values are mean ± SEM as compared to control animals. Asterisks (*, **) indicate values significantly different from each group (P<0.05 and P<0.01). Student's t-test was performed to compare control and treatment groups for all data.

Japan) and then quantified using Image J. Proteins were normalized to β-actin (1:1000), which was used as a loading control.

Statistical analysis

Data were expressed as mean ± SEM and statistically analyzed by the Student's t-test between two groups. Statistical significance was accepted at the P<0.05 level.

Results

Toxicity of agrichemicals in mice

Close observation during 6 hours following intraperitoneal injection in groups of C57BL/6 mice, did not reveal acute poisoning. The color and the lustre of auricular veins were normal, and there were no tremor, no positive Right reflex, no respiratory depression, no convulsions, or salivation changes. Furthermore, no influence on mice body weight was found for any experimental group at any time point (P>0.05) (Figure S1).

Effects of agrichemicals on TH-positive neurons

TH is the rate limiting enzyme of dopamine (DA) synthesis, and a marker for dopaminergic nerve cells. Since both the striata and ventral tegmental area do not have well-defined borders with adjacent brain structures, we defined the boundaries between the two structures with the aid of the mouse brain atlas. As previously described [23], TH-positive neurons were counted if they were stained perceptibly above the background level and only if they contained a nucleus surrounded by cytoplasm. The mean number of TH-positive neurons for each representative mesencephalic section was calculated three times under the final magnification of ×100. The mean of total TH-positive neurons was determined for each mouse from five or six striata values obtained from each animal (**Figure 1**). There were significant decreases (P<0.01) in the number of TH^+ neurons in all treated mice compared with their respective saline and coil controls. We observed a significant decrease in TH^+ neurons in PQ+CPF treated mice compared with the PQ+MB group (P<0.05).

Effects of agrichemicals on α-synuclein

The treatment of mice with PQ+MB failed to cause any significant increases (57.78%, P>0.05) in the expression levels of α-Syn (Mr 17 kDa) in cholate-soluble fractions, compared to the control mice (**Figure 2**). By contrast, PQ caused a significant increase (115.34%; P<0.01) in the levels of α-Syn in the striata, compared to control mice. In mice injected with PQ+CPF, there was a significant increase (154.97%, P<0.01) in α-Syn levels compared to

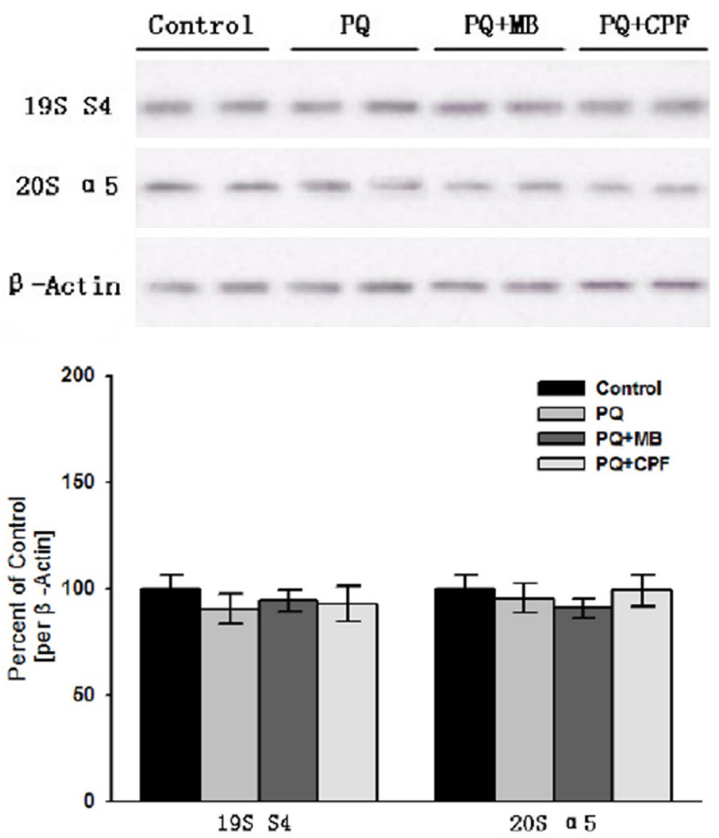

Figure 3. Effects of agrichemicals on ubiquitin-proteasome system (UPS). Samples were homogenized in a modified RIPA buffer containing 1.0% sodium cholate, and analyzed by Western blots. After exposure to initial antibodies, blots were stripped and probed for other proteins. The blots show representative gels while the bar graphs are composites summarized from all animals (n = 5-6). Proteasomal proteins 19S and 20S were probed using antibodies specific for the 19S S4 subunit and 20S α5 subunit, and expressed relative to β-actin used as loading control. Values are mean ± SEM as compared to the saline injected control.

control mice. This increase was significantly different from that obtained using PQ alone ($P<0.05$), which suggests that CPF enhanced the toxicity of PQ, leading to synucleinopathy.

Effects of agrichemicals on UPS

The primary pathway by which proteins are cleared from cells is through proteasomes, thus the increased accumulation of α-Syn could suggest that proteasomal expression may be impaired by the agrichemicals. We then examined the protein levels of representative subunits of both the 19S and 20S components (S4 and alpha 5, respectively) of the 26S proteasome by Western blot analysis (**Figure 3**).

Both the 19S and 20S subunits were detected at similarly reduced levels in all treated mice relative to the control samples. The similar reduction in both the 19S and 20S subunits suggests that there were no substantial alterations in 26S assembly. In paraquat treated mice, 19S S4 levels were decreased by 9.45%, and a decrease of 5.76% was observed in PQ+MB-treated mice. Levels of the 20S alpha 5 subunit in paraquat-treated and maneb + paraquat-treated mice were decreased by 4.66% and 9.22%, respectively. The decrease in S4 and α5 was similar in magnitude to that observed with treatments of PQ alone and CPF alone (7.22% and 1.13%, respectively). However, none of the changes in proteasomal expression levels associated with all treated mice were statistically significant ($P>0.05$). These data indicate that increases in α-Syn observed upon all treatments were not due to the loss of either the 19S or 20S components, or changes in the assembly of the 26S proteasome.

Effects of agrichemicals on autophagic lysosomal pathway

When proteasomes are inhibited, misfolded proteins such as α-Syn start to aggregate and accumulate at nerve terminals, which in turn trigger the activation of the autophagic lysosomal pathway (ALP). Since the effects of these agrichemicals on axonal autophagy were not known, we decided to examine the major components of axonal autophagy in striatal lysates.

In cholate-soluble fractions, PQ significantly increased (47.34%; $P<0.01$) the levels of the mammalian target of rapamycin (mTOR), an overall inhibitor of ALP, and treatment with PQ+CPF augmented the increase in mTOR levels (80.78%, $P<0.01$) (**Figure 4**). Elevated levels of mTOR were also detected in cholate soluble fractions treated with PQ+MB (35.97%, $P<0.05$) (**Figure 4**). Significant ($P<0.01$) increases in levels of beclin 1, an inducer of autophagy, were also observed in cholate-soluble fractions under all the three treatment paradigms, with increases ranging from 81.68 to 95.90%

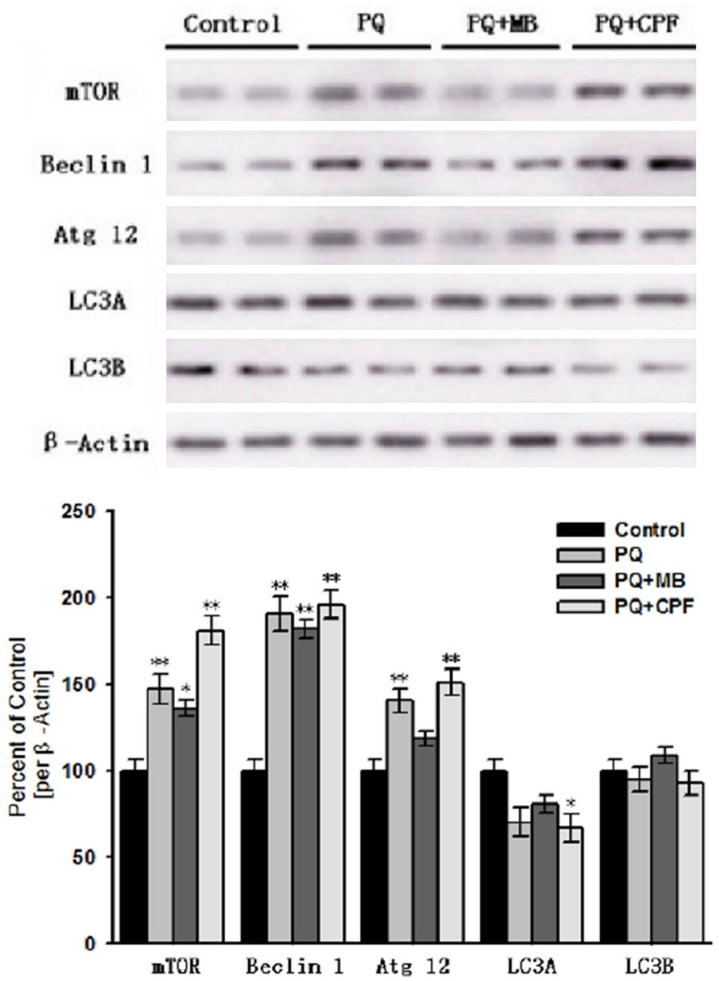

Figure 4. Effects of agrichemicals on the autophagic pathway. Striata from the control, paraqua (PQ), paraqua (PQ) + maneb (MB) and paraqua (PQ) + chlorpyrifos (CPF) injected mice were homogenized in a modified RIPA buffer containing 1.0% sodium cholate, and separated into cholate-soluble and analyzed by Western blots, as described under "materials and methods". The blots show representative gels while the bar graphs are composites summarized from all animals (n = 5-6). Autophagy pathway proteins in the soluble supernatant were probed using antibodies specific for mTOR, Beclin 1, and Atg 12, and expressed relative to β-actin used as loading control. LC3 I (upper band) and LC3 II (lower band in longer exposure blot image) were probed in cholate soluble fractions using LCB antibodies, and the ratio of LC3 II to LC3 I was assessed to measure autophagic flux. Values are mean ± SEM as compared to the saline injected control. Asterisks (*, **) indicate values significantly different from each group (P<0.05 and P<0.01). Student's t-test was performed to compare control and treatment groups for all data.

(**Figure 4**). Levels of Atg 12, another inducer of ALP, were also increased in cholate-soluble extracts of PQ and PQ+CPF-treated striata (40.35% and 50.95%, respectively, P<0.01), but not in PQ+MB-treated animals (**Figure 4**). To gain further understanding of whether autophagosomes are formed, which would enable us to determine whether ALP is inhibited, we examined levels of the microtubule-associated protein 1 light chain 3 (LC3). LC3 is an essential component of the autophagosome (**Figure 4**). In particular, newly synthesized LC3 is proteolytically cleaved to a smaller protein (LC3 I, Mr, 16 kDa) which becomes lipidated (LC3 II, Mr, 14 kDa) and is then inserted into the autophagosome.

The ratio of lipidated LC3-II to cytosolic LC3-I is an important indicator for monitoring the autophagic activity (or flux) [24]. We therefore measured the LC3 II to LC3 I ratio in cholate soluble fractions using the LC3B antibodies (**Figure 4**). It should be noted that the bands of LC3 II are not very stable and are often present as small punctate proteins or faint bands. The calculated ratio was significantly depressed in all the treatment groups (PQ, 25%, P<0.01; PQ+MB, 28%, P<0.05 and PQ+CPF, 22%, P<0.05), indicative of a lowered level of autophagic flux compared to the saline-treated group (**Figure 4**).

Discussion

This study demonstrated, for the first time, that low doses of paraquat and chlorpyrifos induce a broad spectrum of pathological changes in striata. In contrast, the effects of exposure to paraquat and maneb in combination were much milder, causing only a modest inhibition of ALP and a decrease in autophagic flux. Together, these data imply that the combination of paraquat and chlorpyrifos is much more toxic than paraquat alone, inducing changes in striata which mimic those observed in sporadic PD [25], including synucleinopathy, inhibition of proteasomes, and attenuation of ALP.

The mixture of paraquat and chlorpyrifos also caused the increases in α-Syn levels in striata

of mice, but the additional presence of maneb did not augment this increase. The control of 26S proteasome expression is a complex process, whereas our data suggest that all agrichemicals have a mild toxic effect on the regulation of 26S proteasome expression. Small decreases in proteasomal components observed in the paraquat treated, or co-treated, samples could be the result of compensatory changes in response to proteolytic inhibition, which could account for the alleviation of the effect of maneb and chlorpyrifos on 26S expression. However we cannot rule out the possibility that decreases in steady state levels of 26S proteasome in maneb-treated mice could lead to the decreased efficiency of degradation of specific ubiquitinated substrates, which could partially account for the significant toxicity observed in treated mice by the mixture. The toxicity could arise from a direct effect of paraquat on proteolytic activity. The toxicity could also be an effect of meneb or chlorpyrifos on 26S expression, possibly affecting some substrates more than others, which could be an important area of future studies. However, proteasomal inhibition is known to be toxic to cells, as it prevents cells from processing proteins targeted for degradation. Decreases in proteasomal activity have been previously noted in PD substantia nigra [26] and PD striata [25], which could partially account for the both accumulation of α-Syn observed in dopaminergic neurons, interacting with one another and leading to formation of Lewy bodies and Lewy neurites.

Our data suggest that despite increases in beclin 1 and Atg 12, the sharp increases in mTOR may override the stimulatory effects of the other proteins, leading to an overall mTOR-mediated inhibition of ALP in this system. We observed that maneb, chlorpyrifos and paraquat could increase levels of mTOR. Because mTOR is an inhibitor of autophagy, this data suggests that ALP may be dysfunctional in these mice [24, 27-29]. The high levels of mTOR may lead to compensatory increases in levels of other components of the ALP, such as beclin 1, a major initiator of autophagy [24, 28, 29], as well as Atg 12, a marker of the early stages of ALP and a protein important for autophagosome formation. It is noteworthy that in this study we found reduced autophagic flux upon the agrichemical treatment, which is at odds with results reported previously [9]. In this prior work, increases in LC3 proteins were reported to be associated with autophagic vacuoles in cultured SHSY5Y cells treated with paraquat. This could be due to the differences that are intrinsic to *in vitro* versus *in vivo* (our study) models, or, alternatively, could be due to the differences in the effects of acute treatments [9] and that of chronic treatments (our study) on LC3 function. Our Immunocytochemistry (IHC) findings in midbrain regions indicate that impaired autophagy, as indexed by increased levels of mTOR in these regions, is not restricted to striata, but also evident in cell soma.

The effects of environmental toxins on synucleinopathy have been reported only in a small number of studies [8, 15], even though synucleinopathy is a key feature common to most neurodegenerative diseases. In an aging population, the continued widespread use of these agrichemicals could be especially harmful, leading to a higher incidence of diseases such as PD and AD.

Disclosure of conflict of interest

None.

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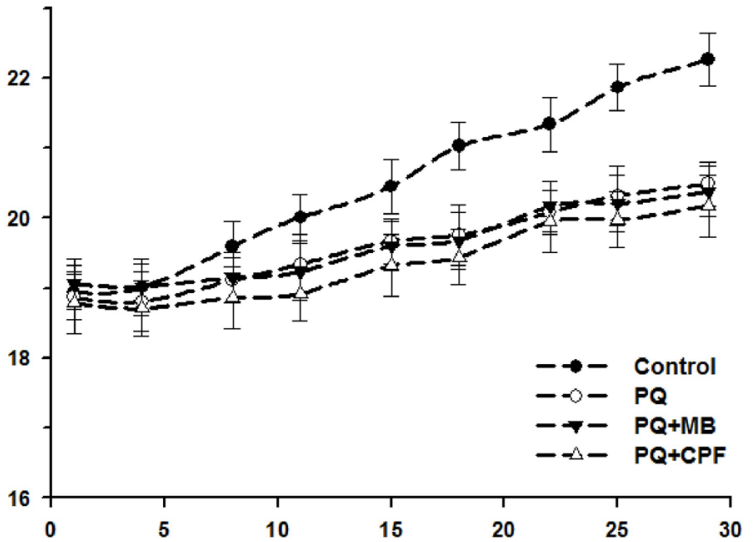

Figure S1. Weight changes in mice treated with paraqua (PQ), maneb (MB), and/or chlorpyrifos (CPF). Over time, there was no significant change in weight following injection of PQ, PQ+MB and PQ+CPF, compared with the control treated mice. The figure shows the lines which are composites of each mice weight summarized from all animals (n = 10-12). Values are mean ± SEM as compared to the control mice.