Objective: To analyze a cluster of 30 industrial coworkers with Parkinson’s disease and parkinsonism subjected to long-term (8–33 years) chronic exposure to trichloroethylene.

Methods: Neurological evaluations were conducted on the 30 coworkers, including a general physical and neurological examination and the Unified Parkinson’s Disease Rating Scale. In addition, fine motor speed was quantified and an occupational history survey was administered. Next, animal studies were conducted to determine whether trichloroethylene exposure is neurotoxic to the nigrostriatal dopamine system that degenerates in Parkinson’s disease. The experiments specifically analyzed complex 1 mitochondrial neurotoxicity because this is a mechanism of action of other known environmental dopaminergic neurotoxins.

Results: The three workers with workstations adjacent to the trichloroethylene source and subjected to chronic inhalation and dermal exposure from handling trichloroethylene-soaked metal parts had Parkinson’s disease. Coworkers more distant from the trichloroethylene source, receiving chronic respiratory exposure, displayed many features of parkinsonism, including significant motor slowing. Neurotoxic actions of trichloroethylene were demonstrated in accompanying animal studies showing that oral administration of trichloroethylene for 6 weeks instigated selective complex 1 mitochondrial impairment in the midbrain with concomitant strionigral fiber degeneration and loss of dopamine neurons.

Interpretation: Trichloroethylene, used extensively in industry and the military and a common environmental contaminant, joins other mitochondrial neurotoxins, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and some pesticides, as a risk factor for parkinsonism.

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Parkinson’s disease is the most common neurodegenerative movement disorder of aging, characterized by progressive motor dysfunctions with features including tremor, rigidity, and bradykinesia. Although genetic influences are undoubtedly important, large cohort twin studies have shown that environmental factors play a crucial role in the development of idiopathic Parkinson’s disease. An understanding of some environmental agents involved began to emerge in the 1980s with the observation that drug abusers and at least one chemist exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its mitochondrial neurotoxic metabolite 1-methyl-4-phenylpyridinium (MPP+) developed clinical and pathological features of Parkinson’s disease. Converging evidence from a number of laboratories implicates mitochondrial dysfunctions in substantia nigra dopamine neurons as a central cause of the disease, leading to the hallmark degeneration of this neuronal population.

In detailed medical histories taken by one of us (K.R.) of 10 Parkinson’s disease patients participating in a phase 1 clinical trial, a patient described chronic industrial exposure to trichloroethylene (TCE), which he suspected to be a factor in his disease. He reported that some coworkers also chronically exposed to TCE had experienced development of Parkinson’s disease. Because there has been increasing concern about the long-term effects of TCE on the nervous system, this study was conducted to clinically evaluate the cluster of Parkinson’s disease patients and their coworkers, and to assess mechanisms of TCE central nervous system neurotoxicity.

A large portion of people have been exposed to TCE through its widespread use as a degreasing agent, inhal-
posed by the National Academy of Sciences noted an association between chronic exposure to TCE and nervous system toxicity that is not well understood.

This study addressed two questions to better characterize the neurotoxicity of TCE. First, we evaluated the clinical features of the Parkinson’s disease patients and 27 coworkers subjected to chronic TCE exposure. Concurrently, experimentation was conducted in 5-month-old male F344 rats to determine whether TCE exposure is neurotoxic to the nigrostriatal dopamine system that degenerates in Parkinson’s disease. Mitochondrial functions were analyzed, as the actions of two known dopaminergic neurotoxins, MPTP and rotenone, involve mitochondrial complex 1 impairment.

Subjects and Methods

Clinical Evaluation of Index Patient and Coworkers

We obtained consent from all subjects in accordance with the University of Kentucky Medical Center Institutional Review Board regulations and guidelines. Neurological evaluations were conducted by one of us (J.T.S.) in the university’s Movement Disorders Clinic on both the index patient and his coworkers with Parkinson’s disease to assess motor functions using standard criteria. Evaluations included a general physical and neurological examination and the Unified Parkinson’s Disease Rating Scale (UPDRS). In addition, an occupational history survey was administered (T.S.P.). Clinical records were available on the one deceased worker. Next, we wanted to determine whether there were movement abnormalities in other former workers exposed to TCE at the factory. A questionnaire was mailed to 134 former workers, of whom 65 responded. Twenty-one individuals self-reported expressing three or more signs of Parkinsonism (slowness of voluntary movement, stooped posture, trouble with balance, slow walk or dragging feet, rigidity or stiffness, tremor, decreased facial expression), 23 respondents reported 1 to 2 symptoms, and 21 reported no symptoms. Fourteen of the 21 workers reporting 3 or more symptoms and 13 coworkers without parkinsonian signs (self-reported) agreed to a clinical examination in the Movement Disorders Clinic. The procedures were identical to those for the index case, with the addition that fine motor hand movement times were measured using an automated Movement Analysis Panel. Informed consent was obtained from all subjects in accordance with the University of Kentucky Medical Center Institutional Review Board regulations and guidelines.

Mitochondria Isolation and Respiration and Enzyme Activity

The methods followed those previously described with some minor modifications. All procedures were performed on ice throughout the protocol. In brief, after CO2 anesthesia, the brain samples and liver were rapidly dissected. The striatal and nigral regions were isolated quickly and carefully using a rat brain matrix. There were nine animals in each treatment group. Three whole-rat substantia nigra were pooled to make one nigral mitochondrial sample, whereas a single whole striatum was used for one striatal sample. Liver tissue dissected from each animal was also used for one mitochondrial sample. The number of samples for the two groups (vehicle and 1,000 mg/day TCE) was three striatum, three substantia nigra, and five liver samples.

Mitochondria were isolated from synaptoneurosomes from the brain samples using nitrogen decompression, and total mitochondria were isolated from the liver samples. Mitochondrial oxygen consumption was measured using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, United Kingdom). Approximately 100 to 150 μg of isolated striatal or nigral mitochondrial protein were suspended and constantly stirred in a sealed and thermostatically controlled chamber at 37°C in respiration buffer (215 mM mannitol, 75 mM sucrose, 0.1% bovine serum albumin, 20 mM HEPES [N-2-hydroxyethyl-piperazine-N’-2-ethane-sulphonate], 2 mM MgCl, 2.5 mM KH2PO4 at pH 7.2). The rate of oxygen consumption was determined based on the response slope of the isolated mitochondria to oxidative substrates, as described previously. State II respiration was initiated by the addition of 5 mM pyruvate and 2.5 mM malate. State III

Analysis of Trichloroethylene Toxicity on Mitochondria and Dopamine Neurons

We conducted studies in 5-month-old adult male Fischer 344 rats to determine whether TCE was a mitochondrial toxin or promoted the loss of midbrain dopamine neurons, or both. Extrapolating from blood levels after oral TCE bolus dosing in rats (best fit with a 2° polynomial: \( r^2 = 0.998; F = 931 \)), peak blood TCE levels would be predicted to run between 55 and 60 μg/ml after a 1,000 mg/kg administration, with a half-life less than 4 hours. Skender and colleagues have reported variable TCE blood levels in workers in dry-cleaning shops, ranging up to 1.7 μg/ml. Although peak TCE blood levels in rats may have been at least 35 times greater than those in typical industrial workers, the goal of the animal study was to replicate in days or weeks what may require years of exposure in humans. Animals received either 1,000 mg/kg TCE in vehicle (0.6% olive oil; Sigma-Aldrich, St Louis, MO) or an equal volume of vehicle by oral gavage. TCE was administered by gavage 5 days/week for 6 weeks; the vehicle control group followed the same dosing regimen. The animals were housed in temperature-controlled rooms and maintained on a 12-hour light/dark cycle. All procedures were conducted in the Laboratory Animal Facilities of the University of Kentucky, which are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The University of Kentucky’s Animal Care and Use Committee approved all protocols.
respiration was initiated by the addition of 150μM adenosine diphosphate followed by the addition of oligomycin (1μg/ml) to inhibit the adenosine triphosphate synthase and induce state IV respiration. The mitochondrial uncoupler carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (FCCP; 1μM) was added to the chamber to induce maximum NADH-linked state V respiration (complex 1 driven). Rotenone (1μM) was added to the chamber to inhibit complex 1 of the electron transport system. Then, FADH maximum respiration (complex 2 driven) was assessed by the addition of succinate (10mM) to the chamber. Three runs were performed for each sample and the average was used for statistical analysis. Respiratory control ratios were calculated for NADH-linked substrates as the ratio of state III/state IV oxygen consumption. All rates are expressed as nanomoles O2 consumed per minute per milligram of protein (O2/min/mg).

Complex I activity was measured in isolated mitochondria as the rotenone-sensitive decrease in NADH absorption at 340nm with ubiquinone-1 as the final acceptor, as described previously20 with some slight modifications.21 In brief, mitochondria were freeze-thawed and sonicated three times, and diluted to 1μg/μl in 10mM KPO4 buffer. The assay was performed in 25mM KPO4 buffer (pH 7.2) containing mitochondrial protein (6μg), 5mM MgCl2, 1mM potassium cyanide, 1mg/ml bovine serum albumin, and 150μM NADH. The reaction was preincubated for 2 minutes at 30°C, the baseline established, and the reaction initiated by addition of coenzyme Q1 (50μM). The activity was measured by monitoring NADH fluorescence (340nm excitation, >450nm emission) over time under the same conditions as described earlier. The assay was also performed in the presence of rotenone (10μM) to determine the rotenone-insensitive activity and the rotenone-sensitive complex I enzyme activity calculated by subtracting the rotenone-insensitive activity from the total activity. All of the assay protocol for a 96-well plate was performed with BioTek Synergy HT plate reader (BioTek, Winooski, VT).

For all analyses of mitochondrial bioenergetics and enzyme activities, the significance of differences among groups was set at p < 0.05 and the data evaluated using an unpaired two-tailed t test.

Nigrostriatal Dopaminergic System Histopathology
Following the same dosing protocols used for the mitochondrial studies, 5-month-old male F344 rats received either vehicle (n = 17) or 1,000mg/day TCE (n = 17) for 6 weeks. The striatum and substantia nigra were recovered from eight animals per group for high-performance liquid chromatography quantification of dopamine and dopamine metabolite levels using procedures described elsewhere.21,22 Three rats from each group received stereotactic injections of the fluorescent tracer fluorogold (Dil; Molecular Probes, Junction City, OR) into four striatal sites.23 This procedure labels neurons in the substantia nigra with axonal projections to the striatum, approximately 90% of which are dopamine neurons.

Tyrosine hydroxylase immunocytochemistry was used to identify dopamine neurons and their processes in the nigrostriatal pathway.24,25 Stereological estimation of the total numbers of tyrosine hydroxylase–positive neurons and fluorogold-labeled neurons was performed by visually counting according to manufacturer’s recommendation (Bioquant, Nashville, TN) using the optical fractionator method, an unbiased quantitative technique independent of size and shape or any conformational changes in the substantia nigra.24,25 In addition, tissue sections from the rostral striatum to the brainstem in the control and TCE-treated rats were histochemically stained for Giemsa (neurons) and Luxol fast blue (white matter), and immunocytochemically stained for α-synuclein.26,27 Data were analyzed by a Student’s t test with a two-tailed distribution. Statistical significance was determined based on a p value less than 0.05. Data are presented as mean ± standard error.

Results
Clinical and occupational histories were collected and evaluated for the index case (Case 1) and coworkers with diagnosed Parkinson’s disease at adjoining workstations (Fig 1A). Diagnosis of PD was based on standard clinical criteria. Individuals had to manifest at least two of the three cardinal signs: tremor at rest, rigidity, and bradykinesia in the absence of a secondary cause such as known neuroleptic/non-TCE toxic exposure. In addition, a clear-cut response to L-dopa or dopamine agonist was required in those individuals meeting clinical criteria. The index case is a 49-year-old man with a 25-year history of occupational exposure to TCE at a small industrial plant employing nearly 300 workers during peak production periods to manufacture small instruments such as metal gauges. The patient worked in a degreasing area with TCE in large vats. He did not use personal protection equipment such as gloves, masks, or an apron. He routinely submersed his hands and forearms bilaterally into the TCE vats while cleaning items being manufactured. Because productivity requirements, he often worked overtime, with workweeks of 65 hours common for 6 months of the year. The major routes of TCE exposure were inhalation and dermal. The patient presented with blepharospasm and “tics” 10 years ago and was diagnosed with Parkinson’s disease 3 years later. In addition to the typical stigmata of Parkinson’s disease, the patient had a marked oral-buccal-lingual impairment, including tongue-rolling and difficulty forming words, which was L-dopa responsive. His other parkinsonian features were also L-dopa responsive (UPDRS score: 60 off L-dopa, 46 on L-dopa). He had a negative family history for parkinsonism.

Case 2 was a white man who died at age 76. He had a 25-year history of occupational exposure to TCE working alongside the index case. They shared the same work experience including the absence of personal protective equipment, work hours, and routes of exposure. This case had a negative medical history aside from the development of Parkinson’s disease, which progressed and contributed to his death 8 years after diagnosis. He presented with disturbed balance,
disturbed cognition, buccal-lingual dyskinesia, and mild tremor 4 years before the diagnosis. He was L-dopa responsive. Signs and symptoms of parkinsonism, once present, were more intense toward the end of workweeks, especially during overtime peaks. This case had two brothers diagnosed with Parkinson’s disease, both of whom experienced much milder courses of the disease.

Case 3 is a 56-year-old woman with a 29-year history of occupational exposure to TCE at the same plant as the first two cases. She shared the same work hours (including overtime) but sat at a work station adjacent to the TCE vat. She received parts cleaned in the vat directly from the first two workers, often wet with TCE. She handled the parts without gloves, thus receiving both dermal and inhalation exposure. She presented with parkinsonian signs and symptoms 5 years ago and was diagnosed with Parkinson’s disease 2 years later. Examination findings included a UPDRS score of 30, bradykinesia, freezing episodes, rigidity, right hand tremor and disequilibrium. She reported worsening difficulties with tongue movements, resulting in moderate communication disturbances. She had an unremarkable medical history, aside from carrying a diagnosis of Parkinson’s disease. Her family history was negative for Parkinson’s disease or other movement disorders.

To determine whether other coworkers exposed to TCE displayed parkinsonian features, we evaluated 14 former employees self-reporting 3 or more parkinsonian signs in response to a mailed survey and 13 workers who self-reported no symptoms (UPDRS, occupational health questionnaire, and timed motor tests13,14). The primary area where each employee worked was mapped (see Fig 1A) showing that the three PD cases worked next to the TCE container for cleaning metal parts. All symptomatic coworkers (Table) worked close to the TCE source. Asymptomatic employees (ie, reporting that they did not have parkinsonian features; see the Table) worked in the same areas or in areas of the factory farther from the TCE vat.

The 14 symptomatic employees ranged from 31 to 66 years of age. They had worked at the factory between 11 and 35 years, presumably exposed to TCE by inhalation during their employment. Although none had Parkinson’s disease based on their clinical examinations, all had positive responses to one or more historical questions on the UPDRS Part I (mentation, behavior, and mood) and Part II (activities of daily living), and/or exhibited signs of parkinsonism on the
UPDRS Part III (motor examination). The symptomatic group was significantly slower ($p < 0.0001$) than age-matched healthy control subjects (see Fig 1B). Also as a group, their fine motor hand movements were slightly faster ($p < 0.01$) than those of the symptomatic group.

While the clinical evaluations were under way, we initiated studies in 5-month-old adult male Fischer 344 rats to determine whether TCE exposure led to central nervous system mitochondria dysfunctions and/or promoted the loss of midbrain dopamine neurons. TCE exposure by oral gavage for 6 weeks significantly inhibited mitochondrial function in the rat substantia nigra (Fig 2A), with mitochondrial state III and V (complex I) functional activity levels significantly lower than that for vehicle-treated control subjects. Both NADH-linked, complex I-driven state III (presence of adenosine diphosphate) respiration and state V (maximum respiration and electron transport system) activity levels were significantly reduced. Because there were no significant differences measured in FADH-linked, complex II–driven state V respiration, TCE administration appeared to be a specifically altering/inhibiting complex I activity. To determine whether TCE treatment resulted in a systemic reduction in complex I–driven respiration, we isolated mitochondria from the striatum, substantia nigra, and liver of the same animals and assessed bioenergetics under the same experimental conditions (see Fig 2B). Significant changes in mitochondrial bioenergetics (respiratory control ratio; state III/IV) were seen only in mitochondria from the substantia nigra. The loss of NADH-driven respiration was further linked specifically to complex I by directly assessing the activity of the enzyme in the substantia nigra, striatum, and liver (see Fig 2C). The results demonstrate that oral TCE administration significantly reduces complex I enzyme activity in the substantia nigra and increases complex I activity in the striatum, perhaps via a compensatory mechanism.

Dopamine neurons in the substantia nigra showed degenerative changes after TCE administration. There was a marked decrease in the number of neurons with the dopaminergic marker tyrosine hydroxylase in both the dorsal and ventral tiers of the substantia nigra in rats administered TCE, whereas dopamine neurons with normal cytoarchitectural features were found in vehicle recipients (Figs 3A, B). The decrease in tyrosine hydroxylase–positive neurons was mirrored in the number of nigral neurons containing fluorogold retrogradely transported from the striatum in TCE recipients (see Figs 3C, D). This visual assessment was confirmed by quantitative cell counts showing significant

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*Human Movement Analysis Panel (hMAP) testing 95% confidence interval for age-matched control subjects was 1.90-2.12 seconds.

UPDRS = Unified Parkinson’s Disease Rating Scale.
losses, with approximately 45% fewer tyrosine hydroxylase–positive neurons present in the substantia nigra of TCE-treated rats (see Fig 3E). Experienced histologists blinded to the treatments could not distinguish differences in Giemsa and Luxol fast blue staining between the two test groups. The evaluations included the cerebellum, with a focus on Purkinje cell layers. However, cytoplasmic α-synuclein–positive inclusions were prominent in the substantia nigra and dorsal motor nucleus of the vagus nerve (dMNX) of TCE recipients. Such inclusions were absent or rare in vehicle recipients. Pathological changes in PD include α-synuclein inclusions in the substantia nigra and the dorsal motor nucleus of the vagus nerve, supporting some specificity of TCE toxicity for the neural circuitry involved in this disease process. The absence of histopathology in Giemsa- and Luxol fast blue–stained sections is not definitive but indicates that there are not major

Fig 2. Chronic administration of 1000 mg/day trichloroethylene (TCE) by oral gavage five days a week for six weeks selectively altered mitochondrial function in five-month old adult male F344 rats. (A) Compared with vehicle treated controls, trichloroethylene significantly reduced Complex I—driven state III and state V activity levels in mitochondrial isolated from the substantia nigra. (B) A typical trace from substantia nigra mitochondrial from trichloroethylene or vehicle-treated animals and illustrates the conditions used to assess mitochondrial bioenergetics. (C) Significant changes in mitochondrial bioenergetics (respiratory control ratio; state III/state IV) were seen only in mitochondrial isolated from the substantia nigra. (D) NADH, a direct measure of complex enzymatic activity, was significantly reduced in the substantia nigra of trichloroethylene-treated animals. The increase in activity in the striatum following chronic trichloroethylene exposure may reflect a compensatory response. Bars are group means, with error bars for the ± s.e.m., * p < 0.01.
disruptions of neurons and fibers of other neural systems. Whereas dopamine levels were significantly reduced in the substantia nigra, striatal dopamine levels were maintained by the surviving neurons. The major impact was on dopamine metabolites (see Fig 3F). The dihydroxyphenylacetic acid/dopamine ratio in the striatum declined significantly \( p < 0.001 \) from 0.176 \( \pm 0.004 \) in the striatum of vehicle recipients to 0.113 \( \pm 0.004 \) in TCE-treated rats, indicating a decrease in dopamine metabolism. Dopamine is metabolized by monoamine oxidase enzymes (types A and B) located on the outer membrane of mitochondria.\(^29\) The decline in the dihydroxyphenylacetic acid/dopamine ratio with TCE treatment is consistent with mitochondrial loss or dysfunction in striatal dopamine neuronal terminals reducing available monoamine oxidase to oxidize dopamine. Homovanillic acid levels were significantly lower in the striatum.

**Discussion**

Converging evidence from a number of laboratories implicates mitochondrial complex I dysfunction as a central event in the degeneration of dopamine neurons in Parkinson’s disease.\(^5,6,30–32\) High levels of mitochondrial DNA deletions have been detected in human substantia nigra neurons in aging and PD using laser microdissection techniques and individual cell polymerase chain reaction.\(^30,31\) Suggesting that mitochondrial dysfunction in nigral dopamine neurons not only increases in aging, but is present before the development of PD. Ekstrand and colleagues\(^32\) have shown that mitochondrial dysfunction alone is sufficient to initiate parkinsonism in conditional knock-out mice by disrupting the gene for mitochondrial transcription factor A in nigral dopamine neurons. Adult mice experienced development of a parkinsonian phenotype with progressive motor dysfunctions. These animal studies demonstrate that the loss of dopamine neurons occurs together with impaired complex I activity in the substantia nigra after TCE exposure. Nigral dopaminergic neurotoxicity has also been reported in mice receiving intraperitoneal injections of TCE.\(^33\) A mechanism for neurotoxic specificity involving complex I inhibition after absorption of TCE in the blood has been de-
scribed involving the formation of 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo). The pathway is TCE → chloral + tryptamine → TaClo. TaClo has been demonstrated to have dopaminergic neurotoxic properties and inhibit complex I activity. TaClo has been measured in blood from patients receiving oral chloral hydrate. The significance is that both chloral hydrate and TCE form chloral in the blood.

It is important to recognize that this study was not a large-scale epidemiological investigation designed to address recall, case finding, or other sources of bias. Nevertheless, these results demonstrate a strong potential link between chronic TCE exposure and parkinsonism. Three coworkers with chronic dermal and inhalation exposure to TCE experienced development of Parkinson’s disease, and many coworkers displayed features of parkinsonism. In France, Guehl and colleagues have reported a case of Parkinson’s disease in a 47-year-old woman after 7 years of exposure to TCE. Three additional Parkinson’s disease patients with a history of chronic industrial exposure to TCE have been described briefly. In this study, clinical evaluations and quantitative motor function testing demonstrated varying degrees of parkinsonism in 27 other employees working near an open vat of TCE and a cluster of three Parkinson’s disease patients. Motor slowing was significantly slower, with fine motor hand performance times up to 250% slower than age-matched healthy control subjects. The average age of the 27 employees was 55 years, which is also the average age of onset of Parkinson’s disease. It will be important to follow the progression of movement disorders in this cohort over the next decade to more fully assess the long-term health risks from TCE exposure.

Whereas group statistical analyses and animal studies have identified pesticides as a risk factor for PD, the primary case studies of PD developing from mitochondrial neurotoxins available before this study were from MPTP exposure. Confounds with MPTP were drug abuse by most individuals and that only a subset of those exposed experienced development of PD, whereas others experienced development of a mild parkinsonism. Likewise, some TCE-exposed individuals experienced development of frank PD, whereas others had only mild-to-moderate parkinsonism. Exposure to TCE at the industrial site was variable and occurred with coexposure to other potential environmental neurotoxins. Other activities at the factory included soldering, welding, and handling metals with toxic potential, which could have contributed to the health problems found in the coworkers. TCE is implicated as a principal risk factor for parkinsonism based on its dopaminergic neurotoxicity in animal models, the high levels of chronic dermal and inhalation exposure to TCE by the three workers with Parkinson’s disease, the motor slowing and clinical manifestations of parkinsonism in coworkers clustered around the TCE source, and the mounting evidence of neurotoxic effects in other reports of chronic TCE exposure. Still, the presence of possible comorbid factors needs to be carefully analyzed and is consistent with Carvey and colleagues’ hypothesis positing that the progressive loss of dopamine neurons characterizing Parkinson’s disease is due to multiple insults leading to the degeneration of the nigrostriatal dopamine system in the brain. We would broaden the hypothesis to include most manifestations of parkinsonism, including dyskinesia.

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