Toll like receptor 4 mediates cell death in a mouse MPTP model of Parkinson disease

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In mammalians, toll-like receptors (TLR) signal-transduction pathways induce the expression of a variety of immune-response genes, including inflammatory cytokines. It is therefore plausible to assume that TLRs are mediators in glial cells triggering the release of cytokines that ultimately kill DA neurons in the substantia nigra in Parkinson disease (PD). Accordingly, recent data indicate that TLR4 is up-regulated by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment in a mouse model of PD. Here, we wished to evaluate the role of TLR4 in the acute mouse MPTP model of PD: TLR4-deficient mice and wild-type littermates control mice were used for the acute administration way of MPTP or a corresponding volume of saline. We demonstrate that TLR4-deficient mice are less vulnerable to MPTP intoxication than wild-type mice and display a decreased number of Iba1 and MHC II activated microglial cells after MPTP application, suggesting that the TLR4 pathway is involved in experimental PD.

Results

We compared MPTP-induced nigrostriatal injury between TLR4-deficient mice and their WT littermates. We observed a significant difference between saline-treated TLR4-deficient mice and WT animals regarding basal dopamine levels (Figure 1A, 72.6 ± 1.5 ng/mg protein vs. 89.3 ± 3.4 ng/mg protein). However, DA metabolites...
were increased in both groups under basal conditions, indicating that dopamine turnover and metabolism were identical regardless of genotype (211.8 ± 5.2 μg/ng vs. 226.0 ± 6.8 μg/ng, Figure 1A).

After MPTP treatment, striatal DA was reduced significantly in both groups; however, there was no significant difference in reduction with regard to genotype (5.9 ± 0.6 ng/mg vs. 9.2 ± 0.8 ng/mg, Figure 1A). The ratio of HVA/DA was significantly increased in both groups after MPTP treatment, reflecting the lesion of the nigrostriatal pathway. However, in mice lacking the TLR4 receptor increase in HVA/DA was significantly less pronounced, indicating a weaker impact of the toxin on cell function in those animals (2611.3 ± 145.0 μg/ng vs. 1458.1 ± 138.1 μg/ng, Figure 1A).

Accordingly, TLR4-deficient mice displayed an increased number of surviving TH+ neurons compared to WT mice (total number of TH+ cells/SN: 6906 ± 394 (WT/saline), 7578 ± 430 (TLR4 deficient/saline), 3828 ± 523 (WT/MPTP) und 5119 ± 326 (TLR4 deficient/MPTP) suggesting that TLR4-deficient mice could be partially protected against MPTP toxicity at the perykaryal level (Figs. 1B and C). However, two-way ANOVA revealed only statistical differences for genotype and treatment but failed to detect a significant interaction between both factors, most likely due to the low power of the test (power for interaction with alpha = 0.05). Since the number of Nissl-positive/TH-negative neurons did not increase in MPTP-treated animals, the decrease in TH-positive neurons could be attributed to cell loss rather than loss of TH protein expression (percentage of TH-negative and Nissl-positive neurons in SNpc; TLR4-deficient mice (97.3% ± 5.2%), WT mice (95.8% ± 4.7%). Furthermore, reduced MPTP metabolism in mutant mice is not likely to account for the observed neuroprotection, as striatal MPP+ content after a single systemic MPTP injection did not differ between genotypes (Supplemental Figure 1).

Finally, to establish a possible link between TLR4 function and microglial cell activation, we assessed the extent of Iba1+ microglial cells within the SN of MPTP-intoxicated WT and TLR4-deficient mice at day 2 and day 7 after MPTP-intoxication. As shown in Fig. 2A there were no differences observed between WT control mice and TLR4-deficient control mice (total number of Iba1+ microglial cells/SN: 1437.5 ± 77.24 (WT/saline), 1392.5 ± 60.14 (TLR4 deficient/saline), but TLR4-deficient mice showed a significantly decreased number of nigral microglial cells after day 2 and day 7 (total number of Iba1+ microglial cells/SN: 5342.5 ± 121.59 (WT, MPTP 2d), 3891.25 ± 154.62 (TLR4 deficient, MPTP 2d); 4786 ±

Figure 1 | (A): HPLC measurement of striatal dopamine (DA) and homovanillic acid (HVA) levels, as well as calculation of the HVA/DA-ratio after MPTP-intoxication of TLR4-deficient (TLR def) and WT mice in comparison to saline treated mice. Values are expressed as mean ± SEM (*p < 0.05 comparing MPTP treated group with its saline control, #p < 0.05 comparing MPTP-treated groups of WT and TLR4-deficient mice. (B): TH-positive cell number in SN after MPTP treatment. WT = WT mice, TLR deficient = TLR4 deficient mice. Values are expressed as mean ± SEM (*p < 0.05 comparing MPTP treated group with its saline control). (C): Representative photographs of the SN of each treatment group is shown. TH-staining of free-floating cryomicrotome-cut sections at day 7. TLR4-deficient = TLR4-deficient mice, WT = wild-type mice. Control = saline treated mice, MPTP = MPTP-treated mice. Scale bars = 200 μm.
88,571,6 (WT/MPTP 7d); 3625 ± 51,17 (TLR4-deficient/MPTP 7d) after MPTP intoxication. In Figure 2C representative photographs of the SN of each treatment group at day 2 are shown.

To demonstrate additional microglia markers we performed MHC II staining at day 2 (Fig. 2B) where no differences were detectable between WT and TLR4-deficient mice (total number of MHC II+ microglial cells/SN: 90 ± 7.98 (WT/saline); 72.5 ± 6.49 (TLR4 deficient/saline). Consistent with the results of the Iba1-staining, TLR4-deficient mice showed a significantly decreased number of nigral activated microglial cells after day 2 compared to WT mice (total number of MHC II+ microglial cells/SN: 440 ± 20 (WT/MPTP) and 190 ± 9.51 (TLR4-deficient/MPTP).

Additionally, we performed a correlation analysis of TH+ and Iba1+ positive cells counts at day 7 where a highly negative correlation coefficient could be calculated between the two variables (r = −0.92, p < 0.001, Fig. 2C).

**Discussion**

Here, we show that TLR4-deficient mice are partially protected against MPTP toxicity which correlates with decreased microglial activation in the SN, suggesting that dopaminergic cell death in this paradigm is, at least to some extent, TLR4-dependent. Sustentative to this result, a recent study observed an augmented expression of TLR4 and CD14 in the SN of mice treated with MPTP in comparison to control animals. Moreover, our group was able to demonstrate upregulation of several TLR (including TLR4) in the MPTP-lesioned SNpc (acute protocol). Additionally, a case-control study showed that a genetic variations within the CD14 monocyte receptor gene (C-260T) was associated with increased risk of PD in females. Both studies underline the potential implication of the TLR4/CD14 pathway in PD pathogenesis.

Drouin-Ouellet et al. were unable to demonstrate a protective effect of MyD88 deletion, the downstream effector pathway to TLR4, against MPTP. However, this group used a subacute MPTP protocol much less likely to induce neuroinflammation than the acute MPTP protocol used in the present study. An alternative, but less likely scenario involves signaling of TLR4 through the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway. Specifically, the TRIF-dependent pathway is able to activate NF-κB, and additionally activates interferon regulatory factor 3 to induce...
tions of the resistance of these mice to MPTP toxicity. Another issue concerns the use of C3H/HeJ mutant mice as a model for TLR4 deficiency. In addition to TLR4, a range of abnormalities have been identified in these mice by quantitative trait locus mapping on chromosomes 4, 6 and 19, which might also account for the altered basal dopamine metabolism compared to C3H/Out mice. These alterations could potentially confound the interpretations of the resistance of these mice to MPTP toxicity.

Interestingly, Stefanova et al. suggest that normal TLR4 function might be necessary to maintain the phagocytic response of microglia to alpha-synuclein, albeit in a multisystem atrophy model where alpha-synuclein is accumulating in oligodendroglia as opposed to neurons. Nonetheless, these data underlie the need for caution when balancing anti- and pro-survival effects of specific receptors and pathways in the context of pathology.

In this respect, it might be postulated that PD-associated pathology reflects a sterile tissue damage (i.e. not mediated by pathogens) condition. In this scenario, brain resident macrophage-like microglia represent important innate immune cells that can be activated by endogenous molecules. Our data suggest that TLR4 is involved in this endogenous activator complex. However, the endogenous activator of TLR4 in this scenario could not be identified yet. A variety of endogenous TLR ligands, for instance heat shock proteins, might contribute significantly to sterile inflammation, and appropriate antagonists may represent a new class of therapeutic agents for such diseases.

We acknowledged that, based on our findings, the contribution of the TLR4-dependent pathway in PD may be modest. Nonetheless, a 10% difference in cell loss can mean the difference between the appearance of suppression of clinical symptoms in patients with early PD. Moreover, therapeutic development in this pathway may not rely on a single, "magic bullet" drug but on a combination of molecules, so-called cocktails. Therefore, exploring the pieces of the puzzle, the different receptors and pathways, does seem a worthwhile effort to us. Further studies will reveal whether pharmacological modulations in the TLR4-pathway could represent a novel therapeutic approach in the treatment of PD.

Methods

Animals. Animals were housed, handled, and cared for in accordance with the Guide for the Care and Use of Laboratory Animals [NCRR (National research council) 1996] and the European Union Council Directive 86/609/EEC, and the experimental protocols were carried out in compliance with institutional ethical committee guidelines (Université Pierre et Marie Curie – UPMC) for animal research.

MPTP intoxication in TLR4-deficient mice. Adult (8–10 weeks) male TLR4-deficient mice and wildtype (WT) littermates (C3H/He and C3H/Out respectively, Jackson Laboratories, USA) were used (n = 3–5 per group at day 7 and n = 4–7 per group at day 2). The co-dominant LPS allele of C3H/He mice was shown to correspond to a missense mutation in the third exon of the Toll-like receptor-4 gene, thereby abolishing TLR4 function. The mice were injected i.p. four times at 2 h intervals with either 25 mg/kg MPTP-HCl or a corresponding volume of saline.

This dose of MPTP resulted from preliminary dose-response results of MPTP metabolism. Animals were sacrificed 2 h and 4 h after this single injection. Neurochemical analysis. Brains (n = 3–5 per group) were rapidly removed and one side of the striatum was used for high-performance liquid chromatography (HPLC) measurement of DA and DA metabolites (3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA)) as previously described. An independent set of experiments, the striatum was dissected for measurement of MPTP metabolism.

Tissue samples were weighted, transferred into 250 ml 0.1 N perchloric acid containing 0.05% sodium metabisulphite and sonicated for 10 sec. After centrifugation at 13,000 g at 4 °C, the supernatants were passed through a 0.2 µm filter (Sartan 13/0.2 RC, Schleicher & Schuell, Germany) and collected for chromatographic assay. The concentrations of DA and its metabolites (DOPAC and HVA) were determined by HPLC coupled with colometric detection. The detector potential was set at 750 mV using a glassy carbon electrode and an Ag/AgCl reference electrode. The detection limits were 1.5 pg for DA and DOPAC and 50 pg for HVA. For MPP+ measurement, striatal tissue was placed into 500 µl 0.1 N HCL04 and were processed as described for catecholamines. MPP+ was assessed by UV detection at a wavelength of 295 nm. Detection limit was 30 pg.

Immunohistochemistry procedures in vivo. The posterior part of the brains were postfixed (4% PFA), and cryoextracted. Immunohistochemistry was performed as described previously on free-floating cryomicrotome-cut sections (20 µm) encompassing the entire midbrain. Sections were incubated with a polyclonal antibody against TH (1:1000; Pel-Freez Biologicals, Rogers, AR) and against ionized calcium-binding adaptor molecule 1 (Iba-1; WAKO, USA; 1: 500), against MHC II as previously described (rat polyclonal anti-major histocompatibility complex class II, Serotec; 1:200). Sections were then treated with secondary antibodies (Vectastain, Vector Laboratories, Burlingame, CA) and subsequently incubated with biotinylated horseradish peroxidase complex. Peroxidase was revealed by incubation with 0.05% 3,3’-diaminobenzidine tetrahydrochloride containing 0.008% hydrogen peroxide, and sections were Nissl counter-stained.

Tyrosine hydroxylase TH + neurons revealed with the chromogen diaminobenzidine were analysed by bright field microscopy using a Nikon Optiphot-2 microscope (Nikon France, Champigny/Marne, France) equipped with the Excel Nova VisualScape 4.18 software (La Rochelle, France) as previously described. TH + cell bodies were quantified stereologically on regularly spaced sections covering the whole mesencephalon from the rostral pole of the substantia nigra to the locus coeruleus using the VisioScan stereology tool. The investigator performing the quantification was blind to the treatment. Tissue blocks from all experimental groups from every animal were processed, and TH + neurons from each individual block were counted using an observer blind to the samples identity. The total number of SN TH + neurons varied from 5860 to 8030 in saline treated WT and 7205 to 8070 in saline treated knock-outs, respectively. For microscopy quantification, similarly adjacent (6–8) serial sections were used. An observer blind to the samples identity quantified the number of Iba1 or MHC II-positive cells in the SN as described above.

Statistical analysis. Data are expressed as mean ± S.E.M. Statistical analysis was performed using two-way ANOVA as genotype and one as saline/MPTP treatment as second independent variable, followed by post-hoc Student-Newman-Keuls test for all pairwise comparisons.

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Author contributions
The work presented here was carried out in collaboration between all authors. C.N., S.H. and A.H. defined the research theme. C.N., S.H. and A.H. designed methods and experiments, carried out the laboratory experiments, analyzed the data, interpreted the results and wrote the paper. L.M., D.A.F., T.L., C.H., D.S., C.D. and L.L. co-designed experiments, discussed analyses, interpretation, and presentation. R.C.D., D.A.F. and E.C.H. analyzed the data and interpreted the results. All authors have contributed, seen and approved the manuscript. A.O., M.B. and P.P.M. co-worked on associated data collection and their interpretation.

Additional information
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Competing financial interests: The authors declare no competing financial interests.

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