Type-1 interferons contribute to the neuroinflammatory response and disease progression of the MPTP mouse model of Parkinson's disease

Bevan S. Main¹, Moses Zhang¹, Kate M. Brody¹, Scott Ayton², Tony Frugier¹, David Steer³, David Finkelstein², Peter J. Crack¹ and Juliet M. Taylor¹

¹Neuropharmacology Laboratory, Department of Pharmacology & Therapeutics, University of Melbourne, Parkville, Melbourne, Australia, 3010

²The Florey Institute of Neuroscience and Mental Health, Parkville, Australia, 3010

³Monash Biomedical Proteomics Facility, Monash University, Clayton, Australia, 3800

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Corresponding author:

Dr Juliet M. Taylor

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Neuropharmacology Laboratory,
Department of Pharmacology & Therapeutics,
University of Melbourne, Parkville, 3010
Melbourne, Australia

Ph: +61 3 9035 7662

Email: juliett@unimelb.edu.au

Main Points:

- Type-1 IFNs are upregulated in human post-mortem Parkinson's disease brains and in the MPTP mouse model
- Genetically or therapeutically targeting type-1 IFN signaling attenuates neuroinflammation and dopaminergic cell death induced by MPTP

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Keywords:

Parkinson's disease, interferons, neuroinflammation, microglia, MPTP

ABSTRACT

Type-1 interferons (IFNs) are pleiotropic cytokines with a critical role in the initiation and regulation of the pro-inflammatory response. However, the contribution of the type-1 IFNs to CNS disorders, specifically chronic neuropathologies such as Parkinson's disease is still unknown. Here, we report increased type-1 IFN signaling in both post mortem human Parkinson's disease samples and in the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) mouse model. In response to MPTP, mice lacking the type-1 IFN receptor (IFNAR1^{-/-}) displayed decreased type-1 IFN signaling, an attenuated pro-inflammatory response and reduced loss of dopaminergic neurons. The neuroprotective potential of targeting the type-1 IFN pathway was confirmed by reduced neuroinflammation and DA cell death in mice treated with a blocking monoclonal IFNAR1 (MAR-1) antibody. The MPTP/MAR-1 treated mice also displayed increased striatal dopamine levels and improved behavioural outcomes compared to their MPTP/IgG controls. These data, implicate for the first time, a deleterious role for the type-1 IFNs as key modulators of the early neuroinflammatory response and therefore the neuronal cell death in Parkinson's disease.

INTRODUCTION

Type-1 interferons (IFNs) are pleiotropic cytokines with a known role in the host immune response to infections and pathogens (de Weerd and Nguyen 2012). They are produced through a number of pathways involving the binding of pathogen associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) to pattern recognition receptors (PRRs) (including the Toll-like receptors (TLRs)). Type-1 IFNs act through the classical JAK/STAT signaling pathway, with subsequent activation of the interferon regulatory factors (IRFs) (IRF-3 and -7) (reviewed in (Ning et al. 2011)), leading to an upregulation in pro-inflammatory cytokine expression, including the type-1 IFNs themselves. In the CNS, both neurons and glial cells are known to produce and respond to type-1 IFNs and increased expression has been linked to a number of CNS disorders (Owens et al. 2013). Specifically, a number of studies suggest that type-1 IFNs have deleterious effects in aging (Baruch et al. 2014) and age-related neurodegenerative disorders such as Alzheimer's disease (Taylor et al. 2014). In this report, we investigated the role of the type-1 IFNs in a mouse model of Parkinson's disease.

Parkinson's disease is the second most common neurodegenerative disorder worldwide, affecting approximately 1% of the population aged 60 years of age (de Lau and Breteler 2006). Pathologically, the disease is characterised by the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). Human studies and animal models of Parkinson's disease have also reported features of neuroinflammation including microglial activation and increased levels of pro-inflammatory cytokines in the SNpc (McGeer et al. 1988; Mogi et al. 1994). However, the initiator of this inflammatory response and its contribution to the DA cell death remain poorly understood.

There is clinical evidence supporting a relationship between the type-1 IFNs and Parkinson's disease including recent reports of patients developing the classical clinical characteristics of the disease following IFN- α administration (Almeida et al. 2009; Bersano et al. 2008; Kajihara et al. 2010; Mizoi et al. 1997). To date however, there is no clear evidence to suggest that type-1 IFNs drive Parkinson's disease progression. For the first time, we demonstrate an involvement of type-1 IFNs in both post mortem human Parkinson's disease brains and in the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease. Importantly, we demonstrate that removal of type-1 IFN signaling either through genetic ablation of the type-1 IFN receptor (IFNAR1) or treatment with a blocking monoclonal IFNAR1 antibody (MAR-1) reduces both the neuroinflammatory response and DA cell death induced by MPTP. The therapeutic potential of targeting the type-1 IFN pathway is further confirmed by improved behavioural outcomes in MPTP-treated wildtype mice administered MAR-1. These data support a deleterious role for the type-1 IFNs as novel modulators of the early neuroinflammatory response in Parkinson's disease contributing to the DA cell death and disease progression.

MATERIALS AND METHODS

Human samples

Human post mortem brain tissue for RNA (frontal cortex) and western blot (SNpc) analysis was obtained from the Victorian Brain Bank Network. Parkinson's disease patients (n=10) and control subjects (n=10) did not differ significantly in terms of their mean age at death (Parkinson's disease patients, 75.80 ± 2.07 years of age, 7 males/3 females; controls, 76.60 ± 2.08 years of age; $p=0.79$, Student's *t* test; mean $\pm$ SEM) or in the mean interval from death to the freezing of tissue (Parkinson's disease patients 34.75 ± 5.05 hours; controls, 46.12 ± 4.19 hours; $p=0.11$, Student's *t* test; mean $\pm$ SEM).

Mice

C57Bl/6 wildtype mice and IFNAR1^{-/-} mice (Hwang et al. 1995) were housed under specific pathogen-free (SPF) conditions according to standard animal care conditions at the Biomedical Research Facility, University of Melbourne.

MPTP treatment regimes

Wildtype mice were injected with four doses of 10mg/kg MPTP (intraperitoneal (i.p); Sigma) or saline (n=3 males) at 2-hour intervals, the total dose per mouse being 40mg/kg. This dose was chosen to elicit ~50% reduction in nigral neurons in the SNpc whilst ensuring all mice would survive the lesioning process (Hung et al. 2012). Mice were killed on day-3, -10 or day-21 post-MPTP by CO₂ inhalation and cervical dislocation before brains were perfused with cold PBS. Hemispheres were removed with mid-brain tissue dissected and snap frozen in liquid nitrogen for protein analysis by western blot or RNA analysis by QPCR.

Wildtype and IFNAR1^{-/-} mice (n=10-12, 5-6 male: females per group) were administered MPTP i.p (4 x 15mg/kg, 2-hour intervals). This dose had been optimised with a new batch of MPTP to elicit •50% reduction in nigral neurons in the SNpc. Mice were killed at day-1, day-3 or day -21 days post-MPTP. Left hemispheres were dissected for protein and RNA with the right hemispheres processed for stereological estimation of SN neurons as previously described (Lei et al. 2012).

To block IFNAR1, an anti-mouse interferon α/β receptor (IFNAR) monoclonal antibody (MAR-1) (1mg) (Leinco Technologies) or IgG isotype control was administered at day-1, day-6 and day-12 post-MPTP by tail vein injection. This dosing regime was chosen to ensure MAR-1 did not influence the metabolism of the toxin and that levels were maintained over the entire MPTP time course, with the half-life of the antibody previously reported (Sheehan et al. 2006). Mice were killed at day-3 post-MPTP for RNA expression and immunohistochemical analysis or day-21 for DA neuron quantitation.

Quantitative Real Time Polymerase Chain Reaction (QPCR)

Mid-brain tissue was homogenised in Trizol reagent (Invitrogen) and RNA isolated as per manufacturers guidelines, with concentration and purity assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). cDNA was synthesised from 1 μ g of RNA using a High-Capacity RNA-to-cDNA Reverse Transcription Kit (Applied Biosystems) as per manufactures guidelines.

The Taqman primers (Applied Biosystems) and SYBR green primers (Geneworks) have been previously described (Taylor et al. 2014). Each sample was performed in triplicate with GAPDH used as an endogenous control gene for normalisation across samples. An ABI Prism 7900HT sequence detection system and SDS 2.4 software (Applied Biosystems) was used to generate threshold cycle (Ct) values for each gene. Fold differences in gene

expression (normalized to control patients or genotype saline control for mice) were calculated by the $\Delta\Delta C_t$ method ($2^{-\Delta\Delta C_t}$) method (Winer et al. 1999). No differences in the GAPDH Ct values between mouse saline and MPTP samples (saline: 18.75 ± 0.324 versus MPTP: 18.03 ± 0.21 , $n=6$) or wildtype and IFNAR1^{-/-} mice (wildtype: 18.91 ± 0.407 versus IFNAR1^{-/-}: 18.48 ± 0.21 , $n=6$) was identified confirming its suitability as a reference gene.

Western blot analysis

Mid-brain tissue was homogenised in Tris buffer (50mM Tris, 150mM NaCl, 1% Triton X-100, Phospho-STOP and Protease inhibitor [Roche]; pH 7.4) and rotated at 4°C for 90 minutes before samples were centrifuged at 2000 x g and supernatants were collected. Protein concentrations of samples were determined by Bradford assay (BioRad) and western blot analysis performed as previously described (Taylor et al. 2014) using the following primary antibodies; phospho-STAT3 (1:1000, Cell Signalling), total-STAT3 (1:1000, Cell Signalling) or β -tubulin or β -actin (1:5000, Sigma). Post-image densitometry was performed using Image J software (NIH), whereby signal intensity was calculated in arbitrary units. For densitometry calculations of human samples, the phosphorylated or total protein intensity was measured in arbitrary units and normalised to the β -actin loading control. A STAT3-P/total STAT3 ratio was then determined from these values and was calculated as a fold change relative to control.

Enzyme-linked Immunosorbent Assay (ELISA)

Murine IL-1 β and IL-6 (BD Biosciences) and IFN α (PBL Interferon Source) ELISAs were performed according to manufacturer's instructions. 100 μ g of protein was loaded per well in duplicate. Protein concentrations of individual samples were determined using a linear curve of mouse IL-1 β , IL-6 or IFN- α standards (4-250pg/mL).

Brain processing, immunohistochemistry and stereology

Following removal, hemispheres were fixed in 5 ml of chilled 4% w/v paraformaldehyde, pH 7.4 (Sigma-Aldrich) overnight at 4°C. They were then left at 4°C overnight in 30% w/v sucrose before being embedded in OCT and cryosectioned into 30µm coronal sections in a 1:3 series of the region encompassing the SNpc.

For immunohistochemistry, sections were permeabilised in 0.2% Triton X-100/PBS (PBS-T) for 20 minutes before being blocked for 1 hour in 10% normal donkey serum/5% BSA/PBS at RT°C. The following antibodies were diluted in 1% BSA and incubated overnight at 4°C; anti-sheep tyrosine hydroxylase (TH) (1:200; Millipore), anti-rabbit Iba1 (1:500, WAKO) and anti-rabbit GFAP (1:500, DAKO). Sections were then washed 3 times in PBS before a 2-hour incubation at RT°C with Alexa Fluor 594-conjugated donkey anti-sheep and Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibodies. Sections were again washed in three washes of PBS before being mounted in Vectashield plus DAPI (Vectashield). Slides were viewed using a Zeiss Axio 123672641 microscope and images captured using an Axio Cam Mrm camera and Zen 2011 software.

For stereology, sections were stained with Tyrosine Hydroxylase (TH) and Neutral Red (Nissl stain; Sigma-Aldrich) and the number of SNpc neurons was estimated using a stereological fractionator design as previously described (Lei et al. 2012). Slides were viewed under an oil objective using a BX51 Olympus Microscope with a design-based stereology software package (Stereo Investigator 7, Microbrightfield, Colchester, VT). The investigator was blinded to the genotype and/or treatment of the animal.

Measurement of MPP+

Striatal tissue samples were prepared by the method of Hows *et al.*, (2004) (Hows et al. 2004). 90 minutes after a single IP injection of 15mg/kg MPTP. Briefly, samples were

homogenised using a Potter-Elvehjem homogeniser in a 0.4M perchloric acid buffer containing sodium metabisulphite (0.1%, w/v), EDTA (0.01%, w/v) and cysteine (0.1%, w/v). Samples were centrifuged for 10 min (10,000 rpm at 4C) and the supernatants frozen for storage before analysis. On analysis, all samples were diluted 1:2 in 0.4M Perchloric Acid Buffer, then centrifuged at 16,000g for 5 minutes and the supernatant was retained for subsequent analysis of MPP+ levels by LCMS.

Dopamine measurement

Dopamine levels were measured in the right striatum by HPLC as previously described (Hung et al. 2012). Peaks were identified by retention times set to known standards. Data were normalized for total wet weight of sample.

Accelerated rotarod test

The accelerated rotarod test was performed as previously described (Lei et al. 2012). Mice were placed on a rotating rod (Panlab, Barcelona, Spain) (4 RPM) with a lane width of 50 mm and rod diameter of 30 mm. Once stabilized, mice were subjected to an incrementally increasing speed of 1 RPM per 8 seconds. Each animal underwent 3 trials on day-20. The length of time that the mice managed to remain on the rod and the speed at which they fell off the apparatus, were recorded. The average of the three trials was used for further analysis.

Pole test

The pole test was performed as previously described (Ogawa et al. 1985), with minor modifications. The mouse was placed head upward on the top of a vertical rough-surfaced pole (diameter 8mm; height 55cm) in their home cage to ensure and encourage the animal's return to a familiar environment. On the day before testing, the animals were habituated to

the pole and were allowed five trials. On the day of testing, the animals completed five trials, during which they were assessed for the time it took to completely turn orientation (T_{turn}) and to descend the pole (T_{latency}). Animals were recorded via a digital video camera on the test day from which time scores were obtained. The times of the 5 trials for both the T_{turn} and T_{latency} were averaged and used in the statistical analysis. In this study, the pole test was performed at 19 days post treatment.

Statistics

All data are expressed as mean $\pm$ SEM. For human QPCR and densitometry, a student's *t*-test was used to compare control and Parkinson's disease or MPTP groups. Mouse QPCR, nigral counts and behavioural data was analysed using a one- or two-way ANOVA, with Dunnett's or Bonferroni *post-hoc* tests where appropriate. For all statistical tests $p < 0.05$ was considered statistically significant.

Study approval

Human post mortem brain tissue was obtained from the Victorian Brain Bank Network, supported by The Florey Institute of Neuroscience and Mental Health, The Alfred and the Victorian Forensic Institute of Medicine and funded by Australia's National Health & Medical Research Council (NHMRC) and Parkinson's Victoria. All procedures were conducted in accordance with the NHMRC National Statement on Ethical Conduct in Human Research (2007), the Victorian Human Tissue Act 1982, the National Code of Ethical Autopsy Practice and the Victorian Government Policies and Practices in Relation to Post-Mortem (Ethics #129/08). All animal studies complied with and were approved by the University of Melbourne, Faculty of Medicine, Dentistry and Health Sciences Animal Ethics Committee (Ethics #1212477).

RESULTS

Type-1 IFN signaling is elevated in post mortem human Parkinson's disease patients

To confirm a role for type-1 IFNs in Parkinson's disease, mRNA expression of IFN- α and IFN- β was analysed by QPCR in post mortem human brain tissue using GAPDH as a control gene (10 Parkinson's disease patients and 10 aged matched controls). IFN- α (Parkinson's disease: 3.21 ± 0.76 -fold versus control: 1.21 ± 0.18 -fold, $n=10$, $p=0.0197$, $df=18$, Student's unpaired t -test) and IFN- β (Parkinson's disease: 4.36 ± 1.05 -fold versus control: 1.11 ± 0.17 -fold, $n=10$, $p=0.0068$, $df=18$, Student's unpaired t -test) mRNA levels were significantly upregulated in Parkinson's disease patients compared to control subjects (Fig. 1A). Type-1 IFNs signal through their cognate receptor, IFNAR, with both receptor subunits, IFNAR1 and IFNAR2 required for signal transduction, however, there was no change in the expression of either IFNAR1 or IFNAR2 in the Parkinson's disease patients (data not shown). Type-1 IFN signaling was further analysed by QPCR and western blot analysis of IRF-7 (Fig. 1A) and phosphorylated (activated) STAT3 (Fig. 1B) levels, respectively. Increased levels of IRF-7 mRNA (Parkinson's disease: 3.00 ± 0.66 -fold versus control: 1.11 ± 0.19 -fold, $n=8$ (control), $n=10$ (Parkinson's disease), $p=0.025$, $df=16$, Student's unpaired t -test) and phospho-STAT3 (Parkinson's disease: 9.65 ± 3.36 -fold versus control: 1.00 ± 0.70 -fold, $n=4$ $p=0.045$, $df=6$, Student's unpaired t -test) were observed in Parkinson's disease patients compared to controls demonstrating there is both increased type-1 IFN ligand expression and signaling in post-mortem Parkinson's disease brains.

MPTP treatment induces a type-1 IFN response in wildtype mice that is reduced in IFNAR1^{-/-} mice

A pilot study was carried out initially in wildtype mice where 4x10mg/kg MPTP was injected i.p every 2 hours. The MPTP mouse model displays an early neuroinflammatory response (Wu et al. 2002) and this occurs prior to marked DA cell loss (Czlonkowska et al. 1996). We detected a significant upregulation in IL-6 mRNA expression at day-3 (2.59 ± 0.23 -fold, $n=3$, $p<0.01$, $df=3$, One-way ANOVA, Dunnett's *post-hoc* test) and day-10 post-MPTP (2.40 ± 0.30 -fold, $n=3$, $p<0.01$, $df=3$, One-way ANOVA, Dunnett's *post-hoc* test) and TNF- α mRNA expression at day-10 post-MPTP (2.10 ± 0.36 -fold, $n=3$, $p<0.05$, $df=3$, One-way ANOVA, Dunnett's *post-hoc* test) (Fig. 2A). QPCR analysis also identified MPTP-induced increased expression of IFN- α (11.88 ± 5.45 -fold at day-3 and 10.69 ± 4.10 -fold at day-10, $n=3$, compared to saline controls) and IFN- β (3.81 ± 1.97 -fold at day-3, $n=3$ and 15.97 ± 0.86 -fold at day-10, $n=3$, $p<0.001$, $df=3$, One-way ANOVA, Dunnett's *post-hoc* test) in wildtype brains (Fig. 2B).

To determine the implications of attenuated MPTP-induced type-1 IFN signaling, 10-12 week old wildtype and IFNAR1^{-/-} mice were administered MPTP (4x15mg/kg) (or saline), before analysis at day-1 or day-3 for neuroinflammatory changes or day-21 for DA cell viability. The ability of IFNAR1^{-/-} mice to metabolise MPTP was comparable to their wildtype counterparts, with no differences in striatal MPP⁺ levels identified as measured by LCMS (wildtype: 1.86 ± 0.08 nmol/mg versus IFNAR1^{-/-}: 2.1 ± 0.1 nmol/mg, $n=3$). Compared to wildtype mice, IFNAR1^{-/-} mice demonstrated significantly reduced levels of the type-1 IFNs, IFN- α (2.33 ± 1.33 -fold versus 21.03 ± 6.27 -fold, $n=6$, $p<0.001$, $df=2$, Two-way ANOVA, Bonferroni's *post hoc* test) and IFN- β (1.49 ± 0.28 -fold versus 3.44 ± 0.78 -fold, $n=6$, $p<0.01$, $df=2$, Two-way ANOVA, Bonferroni's *post hoc* test) (Fig. 3A, B). In addition, expression of IRF-3 (1.53 ± 0.12 -fold versus 0.76 ± 0.04 -fold, $n=6$, $p<0.001$, $df=2$, Two-way

ANOVA, Bonferroni's *post hoc* test), IRF-7 (1.48 ± 0.24 -fold versus 0.61 ± 0.10 -fold, $n=6$, $p < 0.001$, $df=2$, Two-way ANOVA, Bonferroni's *post hoc* test) and IRF-8 (1.56 ± 0.16 -fold versus 1.04 ± 0.10 -fold, $n=6$, $p < 0.01$, $df=2$, Two-way ANOVA, Bonferroni's *post hoc* test) was attenuated in the knockout brains at day-3 (Fig. 3C-E). The reduced mRNA expression correlated with a 1.5- ($n=4$, $p < 0.05$, $df=2$, Two-way ANOVA, Bonferroni's *post hoc* test) and 1.9-fold ($n=4$, $p < 0.001$, $df=2$, Two-way ANOVA, Bonferroni's *post hoc* test) decrease in IFN- α production in the IFNAR1^{-/-} mice compared to their wildtype counterparts at day-1 and -3, respectively, as measured by ELISA (Fig. 3F). IFNAR1^{-/-} mice also exhibited decreased type-1 IFN signaling with reduced MPTP-induced Stat-3 phosphorylation compared to wildtype mice (2.89 ± 0.16 -fold versus 5.37 ± 1.27 -fold, relative to genotype saline control, $n=3$, $p < 0.05$, $df=2$, Two-way ANOVA, Bonferroni's *post hoc* test) (Fig. 3G).

IFNAR1 deficiency results in a reduced neuroinflammatory response following MPTP treatment

In addition to decreased type-I IFN signaling, IFNAR1^{-/-} mice also displayed a reduced pro-inflammatory response, with decreased mRNA levels of IL-1 β (1.82 ± 0.35 -fold versus 1.01 ± 0.08 -fold, $n=6$, $p < 0.05$, $df=2$, Two-way ANOVA, Bonferroni's *post hoc* test) (Fig. 4A) and TNF- α (9.10 ± 2.06 -fold versus 3.21 ± 0.54 -fold, $n=6$, $p < 0.001$, $df=2$, Two-way ANOVA, Bonferroni's *post hoc* test) (Fig. 4B) at day-1 post-MPTP as measured by QPCR. In contrast to wildtype mice, IL-1 β protein levels were also unchanged between saline- and MPTP-treated IFNAR1^{-/-} mice with a 1.6-fold decrease in IFNAR1^{-/-} brains compared to wildtype mice ($n=4$, $p < 0.05$, $df=2$, Two-way ANOVA, Bonferroni's *post hoc* test) at day-1 (Fig. 4C). In addition, the increased number of Iba-1⁺ cells detected in the SNpc of wildtype mice at day-3 post-MPTP was attenuated in the IFNAR1^{-/-} animals (Fig. 4D), supporting a reduced neuroinflammatory response in these animals.

Following CNS insult or injury, it has been suggested that akin to macrophages, resting microglia can polarise into one of two phenotypes, termed 'M1' (classical activation) or 'M2' (alternative activation) (Cherry et al. 2014). It is now generally accepted that microglia exist in a continuum between the two phenotypes and can be differentiated by their expression of specific mediators or cell surface receptors. QPCR analysis of M1 microglial markers supported a pro-inflammatory microglial phenotype induced by MPTP with increased expression in wildtype mice compared to saline controls (Fig. 5A-D). In contrast, expression of M1 markers was relatively unchanged in MPTP-treated *IFNAR1*^{-/-} mice. Levels of CD11b (2.21±0.19-fold versus 1.40±0.11-fold, n=6, p<0.001, df=2, Two-way ANOVA, Bonferroni's *post hoc* test), CD86 (6.00±1.31-fold versus 2.00±0.45-fold, n=6, p<0.001, df=2, Two-way ANOVA, Bonferroni's *post hoc* test), CD32 (3.62±0.63-fold versus 1.65±0.37-fold, n=6, p<0.01, df=2, Two-way ANOVA, Bonferroni's *post hoc* test) and CD16 (1.61±0.26-fold versus 1.01±0.21-fold, n=6, p<0.05, df=2, Two-way ANOVA, Bonferroni's *post hoc* test) were all significantly upregulated in MPTP-treated wildtype mice at day-3 compared to *IFNAR1*^{-/-} animals. In contrast, the expression of M2 markers was not significantly different between wildtype and *IFNAR1*^{-/-} mice following MPTP treatment (Fig. 5E, F).

IFNAR1^{-/-} mice are less susceptible to MPTP-induced dopaminergic cell death

To confirm if this early reduction in the neuroinflammatory response in the *IFNAR1*^{-/-} mice was neuroprotective, quantitation of tyrosine hydroxylase (TH) positive neurons in the SNpc was performed at day-21. In agreement with previous studies, this MPTP treatment regime induced a 53% loss of DA neurons in the SNpc of wildtype mice compared to saline-treated animals (Fig. 6A) (Hung et al. 2012). *IFNAR1*^{-/-} mice displayed attenuated loss of TH⁺ neurons in the SNpc compared to wildtype mice (3907±64.61 versus 3079±70.59, n=10,

$p < 0.001$, $df=1$, Two-way ANOVA, Bonferroni's *post hoc* test), at day-21 post-MPTP. In saline treated mice, no significant difference in the number of TH+ neurons was observed between wildtype and IFNAR1^{-/-} mice (Fig. 6B). In agreement with studies of the neuroinflammatory response induced by MPTP in IFN- γ (Mount et al. 2007) and TNF- α knockout mice (Ferber et al. 2004), we observed a correlation between reduced neuroinflammation and DA cell loss in the IFNAR1^{-/-} mouse.

Administration of MAR-1, an antibody targeted to IFNAR1, is neuroprotective against MPTP neurotoxicity

To investigate the therapeutic potential of targeting the type-1 IFN signaling pathway in our MPTP model, we utilised an IFNAR1 monoclonal blocking (MAR-1) antibody. To ensure MAR-1 was truly neuroprotective *in vivo*, and not just inhibiting the actions of MPTP, administration did not begin until day-1, once the toxin had been metabolized (Meredith et al. 2008). MAR-1 (1mg) was administered at day-1, -6 and -12 following the MPTP treatment regime to maintain inhibition of type-1 IFN signaling (Sheehan et al. 2006). At day-3, QPCR analysis confirmed a MPTP-induced increase in IFN- β (2.02 ± 0.41 -fold) and IRF-7 (1.91 ± 0.09 -fold) in the IgG group compared to their saline counterparts (Fig. 7A, B). This response was attenuated in the MPTP/MAR-1 treated group with both IFN- β (0.72 ± 0.33 -fold) and IRF-7 (0.66 ± 0.07 -fold) levels downregulated relative to the saline/MAR-1 group with a significant difference between the MPTP/IgG and MPTP/MAR-1 groups in terms of both IFN- β ($n=4$, $p < 0.05$, $df=1$, Two-way ANOVA, Bonferroni's *post hoc* test) and IRF-7 ($n=4$, $p < 0.001$, $df=1$, Two-way ANOVA, Bonferroni's *post hoc* test) levels. QPCR analysis also confirmed reduced IL-1 β mRNA levels in the MPTP/MAR-1 treated mice compared to the MPTP/IgG animals (0.72 ± 0.09 -fold versus 1.81 ± 0.25 -fold, $n=4$, $p < 0.01$, $df=1$, Two-way ANOVA, Bonferroni's *post hoc* test) (Fig. 7C). This attenuated pro-inflammatory response

in the MPTP/MAR-1 treated animals was supported by reduced numbers of Iba1+ microglia in the SNpc at day-3, compared to the MPTP/IgG group (Fig. 7D).

In support of the IFNAR1^{-/-} data, reducing type-1 IFN signaling through the administration of the MAR-1 antibody conferred protection against DA cell death at day-21 compared to an IgG control antibody (Fig. 7A). There was no significant difference in the number of nigral neurons between the saline, saline/IgG or saline/MAR-1 treated animals (Fig. 8A, B). MPTP treated mice administered the IgG antibody showed no significant difference in the number of TH+ neurons in the SNpc (3296 ± 109 , $n=10$) compared to MPTP treated animals alone (3250 ± 114 , $n=11$) (Fig. 8B). However, there was a significant increase in the number of TH+ cells in the MPTP/MAR-1 treated animals (3881 ± 193 , $n=11$, $p<0.01$, $df=2$, One-way ANOVA, Dunnett's *post hoc* test) compared to MPTP/IgG animals.

Dopamine levels as measured by HPLC were also significantly elevated in the MPTP/MAR-1 group compared to the MPTP/IgG control animals ($2.9 \pm 0.2 \mu\text{g}/\text{mg}$, $n=15$ versus $2.2 \pm 0.2 \mu\text{g}/\text{mg}$, $n=14$, $p<0.05$, $df=2$, One-way ANOVA, Dunnett's *post hoc* test) (Fig. 8C). Again, there was no significant difference in the levels of dopamine between the saline treated groups (Fig. 8C).

To determine the functional outcomes of decreased DA cell death, mice were subjected to the rotarod and pole test performance tests at day-19 and -20. Established behavioural testing protocols that required minimal training and handling were employed to minimise stress on the animals and therefore potential influences on DA release (Bergquist et al. 2003). As expected, MPTP treated mice performed worse than their saline counterparts with a reduction in the latency to fall time (85.76 ± 6.61 seconds, $n=15$ versus 61.97 ± 5.59 seconds, $n=15$, $p=0.0103$, Two-tailed *t*-test) in the rotarod analysis (Fig. 7D). Although not significant, the MPTP/MAR-1 group did display improvements in the rotarod analysis compared to the MPTP/IgG controls (74.62 ± 4.49 seconds, $n=16$ versus 65.58 ± 1.94 seconds,

n=13). In addition, whilst there was a significant difference between the MPTP/IgG and saline/IgG groups (65.58 ± 1.94 seconds, n=13 versus 80.42 ± 3.13 seconds, n=11, $p < 0.05$, $df=3$, One-way ANOVA, Bonferroni's *post hoc* test), the latency to fall between the MPTP/MAR-1 and saline/MAR-1 animals (74.62 ± 4.48 seconds, n=16 versus 80.76 ± 2.49 seconds, n=11) was not significantly different, supporting a neuroprotective effect of MAR-1. MPTP treated mice also performed worse at the pole test compared to saline animals (4.54 ± 0.54 seconds, n=14 versus 2.47 ± 0.19 seconds, n=14, $p = 0.0012$, Two-tailed *t*-test) (Fig. 8E). The MPTP/MAR-1 treated mice displayed improvements in the pole test in terms of time to turn when compared to their MPTP/IgG counterparts (Fig. 8E). MAR-1 protected against the MPTP-induced increase in time to turn seen in the IgG control group (saline/IgG; 2.55 ± 0.02 seconds, n=12 versus MPTP/IgG; 4.00 ± 0.27 seconds, n=12, $p < 0.001$, $df=3$, One-way ANOVA, Bonferroni's *post hoc* test) with no significant difference between the saline and MPTP treatment groups (saline/MAR-1; 2.58 ± 0.26 seconds, n=11 versus MPTP/MAR-1; 3.02 ± 0.26 seconds, n=11). In addition, there was a significant improvement between the time to turn of the MPTP/IgG and MPTP/MAR-1 treated animals (4.00 ± 0.27 seconds, n=12 versus MPTP/MAR-1; 3.02 ± 0.26 seconds, n=11, $p < 0.05$, $df=3$, One-way ANOVA, Bonferroni's *post hoc* test).

DISCUSSION

For the first time, we have confirmed increased mRNA expression of the type-1 IFNs and their downstream mediators, in post-mortem Parkinson's disease brains. The type-2 IFN, IFN- γ has previously been implicated in Parkinson's disease with increased blood plasma levels reported (Mount et al. 2007), however our data is the first evidence of elevated levels of the type-1 IFNs in human condition. It is interesting to note that there seemed to be a dichotomisation of groups with the QPCR analysis, however we were unable to confirm the cause of this due to the small number of post-mortem brain samples we were able to access.

A greater sample size, with the associated clinical data and genetic analysis is required to determine if the type-1 IFNs correlate with disease severity, or potentially a particular genetic mutation such as LRRK2 (Gillardon et al. 2012; Kim et al. 2012; Moehle et al. 2012) or α -synuclein (Gao et al. 2011; Roodveldt et al. 2008; Zhang et al. 2005).

There are a number of toxin-based models used to mimic the human condition, each with their strengths and limitations. Consistent with Parkinson's disease in humans, MPTP produces the specific loss of DA neurons in the SNpc, damage to the DA nigrostriatal pathway and neuroinflammatory changes including increased pro-inflammatory cytokine expression, microglial activation and astrogliosis (Czlonkowska et al. 1996; Smeyne and Jackson-Lewis 2005). Our study did confirm an early neuroinflammatory response in wildtype mice including increased type-1 IFNs at day-3, a response that was attenuated in the IFNAR1^{-/-} animals. Evidence suggests that the initial expression of the type-1 IFNs is through an IRF-3- then IRF-7-dependent mechanism (Honda et al. 2005), with expression of both IRFs reduced in the knockout animals. The expression of activated (phosphorylated) Stat-3 was also significantly reduced at day-3 in the IFNAR1^{-/-} mid-brains. Stat-3 has been

shown to negatively regulate Stat-1 dependent inflammatory responses induced by the type-1 IFNs (Ho and Ivashkiv 2006). It could be postulated that this negative feedback loop is activated in the wildtype animals to dampen the inflammatory response, including the type-1 IFNs that is induced by MPTP. In contrast, the IFNAR1^{-/-} animals, which are unable to signal through the classical JAK-STAT pathway, display significantly lower levels of phospho-Stat3, potentially through an alternative signaling cascade, for example, that involving IL-6 (Beurel and Jope 2009)

It was interesting to note that, unlike the wildtype brains, IRF-8 levels were unchanged in the IFNAR1^{-/-} mice. It has been recently reported by a number of *in vitro* and *in vivo* studies that IRF-8 is a critical mediator of the reactive microglial phenotype in the CNS (Masuda et al. 2012; Minten et al. 2012). Our study suggests MPTP does induce a more “M1-like” microglial phenotype in wildtype mice with increased expression of CD11b, CD86 and CD16/32 and low levels of IL-10 (data not shown), and elevated levels of classical pro-inflammatory cytokines TNF- α and IL-1 β . This microglial signature was attenuated in the knockout animals with the reduced number of Iba1⁺ cells in the SNpc of IFNAR1^{-/-} mice supporting this. In contrast, although MPTP did upregulate the expression of markers associated with the anti-inflammatory M2a phenotype, this was not nearly as significant, with no difference between the wildtype and IFNAR1^{-/-} mice. The use of “M1” or “M2” nomenclature to describe microglia (and macrophages) has been questioned recently with it suggested that microglia exist rather as a continuum of the two or as a mixed phenotype (reviewed in (Moehle and West 2015)). The “switching” of microglia to a more “M1-like” response has been reported to be IRF-7-mediated (Tanaka et al. 2015) and we did see an increased MPTP-induced IRF-7 expression in the wildtype but not IFNAR1^{-/-} mice. The decreased expression of M1 markers together with the reduced microglial activation and levels of TNF α and IL-1 β in the IFNAR1^{-/-} brains is all supporting evidence for an

attenuated pro-inflammatory response in these mice. Type-1 IFN-IFNAR1 signaling mediates the pro-inflammatory response through the IRFs which are also known to induce type-1 IFN expression in a positive-feedback mechanism (Ikushima et al. 2013). This modulation has been confirmed in a number of *in vitro* and *in vivo* models including those of our own (Cox et al. 2015; Karve et al. 2016; Taylor et al. 2014) and both IRF-3 and IRF-7 were upregulated in MPTP-treated wildtype mice.

This attenuated early neuroinflammatory response in the IFNAR1^{-/-} brains was associated with reduced MPTP-induced DA cell loss at day-21. This restoration in nigral cells is consistent with studies with IFN- γ and TNF receptor knockout mice that displayed reduced neuroinflammation and MPTP-induced cell loss (Mount et al. 2007; Sriram et al. 2002). Recently, it has been reported that TLR4 knockout mice display an attenuated loss (13% compared to wildtype mice) of DA neurons in the MPTP model (Noelker et al. 2013). In addition, intrastriatal injection of LPS, a Gram-negative bacterial endotoxin that induces an inflammatory response through TLR4, has also been shown to induce specific loss of DA neurons within the SN (Hunter et al. 2009). Critically, the binding of LPS to the TLR4 receptor induces type-1 IFN production and we have confirmed this occurs *in vitro* in wildtype but not IFNAR1^{-/-} glia (unpublished data). Although the type-1 IFNs were not measured in either of these studies, it does support our findings that an early pro-inflammatory response is detrimental to DA cell viability, a response influenced by pathways involving the type-1 IFNs. In turn, our data supports recent studies implicating the type-1 IFNs in the cognitive decline in aged mice and also in the neuroinflammatory response and amyloid pathology present in the J20 transgenic mouse Alzheimer's disease model (Baruch et al. 2014; Mesquita et al. 2015). Significantly, the increase in type-1 IFN expression preceded any amyloid pathology in the J20 mice. Contrary to this detrimental role for elevated type-1 IFNs in Parkinson's and Alzheimer's disease (Taylor et al. 2014) it has recently been

reported that mice lacking the type-1 IFN, IFN- β , develop spontaneous neurodegeneration with motor and cognitive learning impairments (Ejlerskov et al. 2015). However, we failed to see any significant differences between wildtype and IFNAR1^{-/-} saline animals in terms of DA cell number or TH staining (data not shown), nor dopamine levels or behaviour in saline/MAR-1 treated mice, suggesting under our basal housing conditions, these are not influenced by decreased IFN-IFNAR1 signaling. It is interesting to note, that unlike our study Ejlerskov *et al.* (2015) did not address the neuroinflammatory response, the contribution of the glial cells nor levels of IFN- α in the context of reduced IFN- β signaling. Indeed, it highlights the complexity of this pathway in terms of α/β subtypes and in specific cells of the brain.

Therapeutically targeting the neuroinflammatory response has shown some neuroprotective benefits in both *in vitro* and *in vivo* models of the disease (Arai et al. 2004; Du et al. 2001; McCoy et al. 2006). Epidemiological evidence also supports a role for the neuroinflammatory response in Parkinson's disease progression with protective effects of chronic NSAID reported (Samii et al. 2009). We chose to therapeutically target the type-1 IFN pathway by blocking signaling through IFNAR1. The monoclonal blocking antibody, MAR-1, has been well characterised in terms of its ability to attenuate the type-1 IFN response *in vitro* and *in vivo* (Sheehan et al. 2006). It has recently been used to attenuate the age-related increase in the type-1 IFNs with subsequent improvements in cognition and neurogenesis in mice (Baruch *et al.*, 2014) and we have also reported its neuroprotective effects in a model of traumatic brain injury (Karve et al. 2016). In the current study, MAR-1 attenuated both the type-1 IFN response and microglial activation in the SNpc at day-3 and provided some neuroprotective effects against MPTP-induced cell death with improvements in motor-coordination at day-21. Although we did confirm the ability of MAR-1 to reduce both the early type-1 IFN and neuroinflammatory response in the MPTP brain, the target of

MAR-1, in terms of its ability to limit the production of the type-1 IFNs from a particular cellular source, still needs to be identified. We chose to administer MAR-1 via an i.v tail vein injection however BBB disruption has been reported in both PD patients (Kortekaas et al. 2005) and in animal models of the disease, including MPTP (Chung et al. 2013). We postulate that the microglia (Hamner et al. 2015) and even the dying DA neurons themselves (releasing DAMPs) are both the producers and targets of the type-1 IFNs. In support of this, our *in vitro* studies (unpublished) with primary cultured neurons and glia demonstrate that MAR-1 is able to attenuate the type-1 IFN response induced by the Parkinson's disease toxin, rotenone, in both cell types. However, we cannot rule out the possibility that peripheral cells are the primary source of IFNs and therefore the targets of MAR-1. The infiltration of CD4+ and CD8+ T cells in the PD brain has been demonstrated (Brochard et al. 2009). In addition, MHC II null mice display attenuated microgliosis and astrogliosis with reduced MPTP-induced DA cell loss supporting a role for the adaptive immune response in the disease progression (Martin et al. 2016). Therefore the influence of the type-1 IFNs on the peripheral immune response in Parkinson's disease warrants further study. We believe that modulation of the type-1 IFN pathway to address the early neuroinflammatory response in Parkinson's disease may slow the disease process. A greater understanding of the neuroinflammatory processes contributing to Parkinson's disease pathology will aid the development of new approaches in order to slow the progression of this debilitating disorder.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Type-1 signaling is upregulated in post-mortem Parkinson's disease brains.

(A) Increased mRNA expression of IFN- α , IFN- β and IRF-7 in post-mortem Parkinson's disease brains (n=10) compared to controls (n=10). Data represents mean $\pm$ SEM, *p<0.05, **p<0.01. (B) Increased phosphorylated STAT-3 (STAT3-P) in the SN of Parkinson's disease brain (n=4) compared to controls (n=4). Levels of STAT3-P and Total STAT3 levels were normalised to β -actin levels. A STAT3-P/Total STAT3 ratio was then determined from these values and was calculated as a fold change relative to controls. Data represents mean $\pm$ SEM, *p<0.05.

Figure 2. MPTP induces a proinflammatory response, including increased type-1 IFN expression *in vivo*.

mRNA expression of TNF- α and IL-6 (A) and IFN- α and IFN- β (B) in mid-brain tissue from mice treated with saline (n=3 mice) or MPTP (4x10mg/kg) (n=3 mice for each timepoint). Data represents mean $\pm$ SEM, *p<0.05, **p<0.01, ***p<0.001 and is expressed relative to saline control.

Figure 3. IFNAR1^{-/-} mice display reduced MPTP-induced type-1 IFN signaling.

Type-1. IFN- α (A), IFN- β (B), IRF-3 (C), IRF-7 (D) and IRF-8 (E) mRNA levels were determined by QPCR analysis (n=6 for each time-point). (F) Levels of IFN- α were measured by ELISA in WT and IFNAR1^{-/-} brains (n=4). (G) Phosphorylated Stat3 (Stat3-P) was detected in wildtype and IFNAR1^{-/-} brains (n=3) by western blot. Levels of Stat3-P and Total Stat3 levels were normalised to β -actin levels. A Stat3-P/Total Stat3 ratio was then determined from these values and was calculated as a fold change relative to genotype saline control. Data is expressed as mean $\pm$ SEM, *p<0.05, **p<0.01, ***p<0.001.

Figure 4. IFNAR1^{-/-} mice display a reduced neuroinflammatory response induced by MPTP. (A) mRNA expression of IL-1 β and TNF- α was analysed by QPCR in mid-brain regions from wildtype (WT) and IFNAR1^{-/-} mice (n=6) mice. Data expressed relative to genotype saline control. IL-1 β protein levels were also decreased in the IFNAR1^{-/-} mice as measured by ELISA (n=4). All data is expressed as mean $\pm$ SEM, *p<0.05, **p<0.01, ***p<0.001. **(B)** Representative images (n=3 mice for each timepoint) demonstrating reduced Iba1+ cells (green) at day-3 in the SNpc of IFNAR1^{-/-} brains (Panels D–F) compared to wildtype (Panels A–C) brains, suggesting decreased microglial activation. Panels C and F are inserts of Panels B and E, respectively. Sections co-labelled with the DA neuronal marker, TH (red). Scale bar = 100 μ M.

Figure 5. Expression of M1 microglial markers induced by MPTP is reduced in IFNAR1^{-/-} mice

(A–D) mRNA expression of the pro-inflammatory M1 microglial markers CD11b, CD86, CD32 and CD16. **(E, F)** Mid-brain mRNA expression of the M2 markers YM1 and CD206. Data expressed relative to genotype saline control, n=6 for each genotype at each time-point and is expressed as the mean $\pm$ SEM, *p<0.05, **p<0.01, ***p<0.001.

Figure 6. Reduced loss of dopaminergic neurons in MPTP-treated IFNAR1^{-/-} mice. (A) Representative images of TH-stained sections through the mid-brain (SN and VTA) in saline- or MPTP-treated wildtype and IFNAR1^{-/-} mice at day-21. Scale bar = 250 μ M. **(B)** The number of TH+ neurons in the SN was quantitated by unbiased stereology. Data is expressed as mean $\pm$ SEM, n=10, ***p<0.001.

Figure 7. The MPTP-induced type-1 IFN and neuroinflammatory response is attenuated by MAR-1 *in vivo*

(A-C) mRNA expression of IFN- β , IRF-7 and IL-1 β in MAR-1 treated and IgG control animals as determined by QPCR. All data expressed relative to their respective saline control and as the mean $\pm$ SEM, n=4, *p<0.05, **p<0.01, ****p<0.0001. (D) Representative images (n=3) demonstrating reduced Iba1+ cells (green) at day-3 in the SNpc of MPTP/MAR-1 brains compared to IgG/MAR-1 brains, suggesting decreased microglial activation. Sections co-labelled with the DA neuronal marker, TH (red). Scale bar = 100 μ M.

Figure 8. Administration of the IFNAR1 blocking antibody, MAR-1 is neuroprotective in the MPTP mouse model of Parkinson's disease. (A, B) TH+ staining and quantitation by unbiased stereology of saline-treated or MPTP-treated animals administered either MAR-1 or an IgG control antibody. Data is expressed as mean $\pm$ SEM, n=7-8 for each saline group, n=10-11 for each MPTP group, **p<0.05. (C) HPLC identified increased dopamine levels in the MPTP/MAR-1 treatment group (compared to the MPTP/IgG or MPTP alone). Data is expressed as mean $\pm$ SEM, n=13, *p<0.05. Improvements in the performance in both the rotarod (D) (mean $\pm$ SEM, n=11-16, *p<0.05) and pole test (E) (mean $\pm$ SEM, n=11-14, *p<0.05, **p<0.01, ***p<0.001) were identified in the MPTP/MAR-1 treated animals compared to the MPTP/IgG controls.

Figure 1
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Figure 2
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Figure 3
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Figure 4
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Figure 5
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Figure 6
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Figure 7
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Figure 8
209x297mm (300 x 300 DPI)

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Author/s:

Main, BS;Zhang, M;Brody, KM;Ayton, S;Frugier, T;Steer, D;Finkelstein, D;Crack, PJ;Taylor, JM

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