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Type-I interferons mediate the neuroinflammatory response and neurotoxicity induced by rotenone

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Abbreviations: AD, Alzheimer's disease; DA, dopaminergic; GFAP; DAMPS, damage-associated molecular patterns; Glial fibrillary acidic protein; IFN, interferon; IFNAR1, interferon receptor 1; IL; interleukin, MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTT, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PD, Parkinson's disease; SNpc, substantia nigra pars compacta; TLR; toll-like receptor; TNF, tumour necrosis factor.

Abstract

Evidence from post-mortem human brains, animal studies and cell culture models has implicated neuroinflammation in the aetiology of chronic neuropathologies including Alzheimer's and Parkinson's diseases. Although the neuroinflammatory response is considered detrimental in contributing to these pathologies, the underlying mechanisms are still not well understood. The type-I interferons (IFNs) have been well characterised in the periphery and are known to initiate/modulate the immune response. Recently, they have been implicated in aging and we have also demonstrated increased type-I IFN expression in post-mortem human Alzheimer's and Parkinson's disease brains. We hypothesise that the type-I IFNs are key drivers of the damaging, self-perpetuating pro-inflammatory response that contributes to these chronic neuropathologies. In support of this, we have recently confirmed in models of Alzheimer's and Parkinson's disease that mice lacking the type-I IFN receptor (IFNAR1), display an attenuated neuroinflammatory response with subsequent neuroprotection. To further investigate type-I IFN mediated neuroinflammation and the

specific CNS cell types involved, this study treated primary cultured wildtype and IFNAR1$^{-/-}$ neurons or mixed glia with the mitochondrial complex I inhibitor, rotenone. Wildtype neurons and glia treated with 3nM and 25nM rotenone, respectively exhibited a pro-inflammatory response, including increased type-I IFN expression that was attenuated in cells lacking IFNAR1. Reduced type-I IFN signaling in IFNAR1$^{-/-}$ neurons also conferred protection against caspase-3 mediated rotenone-induced cell death. Further, this reduced pro-inflammatory response in the IFNAR1$^{-/-}$ glia subsequently diminished their neurotoxic effects to wildtype neurons. In support of this, we confirmed that therapeutically targeting the type-I IFN glial response to rotenone through a specific IFNAR1 blocking monoclonal antibody was neuroprotective. Our data has confirmed that both neurons and glia contribute to the pro-inflammatory response induced by rotenone with attenuation of this response beneficial in reducing neuronal cell death.

Introduction

Interferons (IFNs) are pleiotropic cytokines with a known role in the host immune response to infections and pathogens (de Weerd & Nguyen 2012). Type-I IFNs (IFN-α and IFN-β) exert their effects through the classical Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) signaling pathway, leading to the secretion of classical pro-inflammatory cytokines (including Tumour Necrosis Factor-α (TNF-α), Interleukin-6 (IL-6) and Interleukin1-β (IL1-β)) and induction of apoptosis. Indeed, without the presence of type-I IFNs an innate inflammatory response is unable to be generated (de Weerd & Nguyen 2012). In the CNS, both neurons and glial cells are known to produce and respond to type-I IFNs (Owens *et al.* 2013) and increased type-I IFN expression has been linked to CNS disorders including systemic lupus erythematosus (Meyer 2009), HIV-encephalopathy (Rho *et al.* 1995) and Aicardi-Goutieres syndrome (Crow *et al.* 2006). More recently, the type-I IFNs have ben linked to the elevated neuroinflammatory response in the lysosomal storage disease, Gaucher disease (Vitner *et al.* 2016).

Recently, a role for the type-I IFNs in aging has also been proposed. Specifically, a type-I IFN dependent gene expression profile was reported in the choroid plexus of aged mice and humans (Baruch *et al.* 2014). Blocking of type-I IFN signaling restored cognitive function and hippocampal neurogenesis in aged mice. We have recently confirmed increased type-I IFN levels in post-mortem human AD brains and in the APP/PS1 mouse (Taylor *et al.* 2014). Furthermore, mice with attenuated type-I IFN signaling (APP/PS1 mice crossed with IFNAR1$^{-/-}$ mice) display an altered neuroinflammatory phenotype with reduced microglial activation and some cognitive improvement at 9 months of age (Minter *et al.* 2016a). Recently, a number of animal models have also supported a detrimental role for type-I IFN signaling in PD. Qin *et al.*, (2016) demonstrated a type-I IFN signature in an alpha-synuclein rat model, with attenuation of the downstream JAK-STAT signaling protecting against DA neurodegeneration (Qin *et al.* 2016). We have confirmed that genetically or pharmacologically blocking the IFN receptor, IFNAR1, to diminish type-I IFN signaling attenuates the early neuroinflammatory response in the MPTP mouse model of PD (Main *et al.* 2016). These mice displayed reduced levels of pro-inflammatory cytokines and microglial activation, which correlated with decreased MPTP-induced nigral degeneration and improvements in striatal dopamine levels and behavioural outcomes. These studies suggest that type-I IFNs have deleterious effects in age-related neurodegenerative disorders.

In this study, we used an *in vitro* rotenone model to further characterise the cellular source and contribution of the type-I IFNs to the neuroinflammatory response that contributes to neuronal cell death. Rotenone is a mitochondrial complex I inhibitor known to be neurotoxic to both dopaminergic (Choi *et al.* 2015) and non-dopaminergic neurons (Chen *et al.* 2006), with this attributed in part to its effects on mitochondrial respiration and elevated oxidative stress. Significantly, in studies with primary cultured glia/microglia it has also been shown to induce oxidative stress and a neuroinflammatory response, including increased pro-inflammatory cytokine secretion (Ye *et al.* 2016). We hypothesise that the type-I FINs are critical mediators of rotenone-induced neuroinflammation and neuronal cell death. Here we present evidence for a critical role of the type-I IFNs in mediating the neuroinflammatory response induced by rotenone *in vitro* in both primary cultured neurons and glia. Importantly, we demonstrate that the attenuation of type-I IFN signaling is neuroprotective in this model confirming the crucial role these cytokines play in mediating the neuroinflammatory response that contributes to neuronal cell death in chronic neuropathologies.

Methods

Mice

IFNAR1$^{-/-}$ mice were initially generated by (Hwang *et al.* 1995) with wildtype C57Bl/6 time-mated female mice sourced from Australian Resources Centre (ARC, Western Australia). All pregnant female mice were housed under specific pathogen-free (SPF) conditions in a 20°C to 24C room with a 12 h light/dark cycle and with food and water *ad libitum* according to standard animal care conditions at the Biomedical Research Facility, University of Melbourne. 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).

Isolation of mixed cortical and hippocampal neurons

Mixed cortical and hippocampal neurons were isolated from day 14-16 embryos as previously described (Crack *et al.* 2006, Taylor *et al.* 2005). The purity of cultures was determined to be >95% neurons as indicated by Fox-3/NeuN and GFAP staining to identify neurons and glia, respectively. Neuronal cultures were utilised for treatment at 9 days *in-vitro*.

Primary mixed cortical and hippocampal glial cultures

Mixed primary glial cells were isolated from post-natal P0-P1 C57BL/6 wildtype and IFNAR1$^{-/-}$ genotypes as previously described (Minter et al. 2016a). Cells were cultured in T75cm^2 flasks in DMEM (containing 20% FBS, 0.5% penicillin-streptomycin). DMEM culture medium was changed every 3-4 days and cells cultured until reaching approximately 90-95% confluency. Cells were used at passage 2-3 and were plated into 10cm dishes for experimental use.

Cell treatments

Rotenone (Sigma-Aldrich) was prepared fresh for each experiment, to a 12.7µM stock solution in dimethylsulphoxide (DMSO), which was diluted further to desired final concentrations in treatment medium before use (final concentration of DMSO <0.001% v/v). For MTT and caspase-3 assays, cells were treated with vehicle or rotenone for 24h or 8, 24 and 48h, respectively. For mRNA analysis, neurons and glial cells were treated over a time course with either 3nM or 25nM rotenone, respectively.

MAR1-5A3 (monoclonal antibody specifically targeted to block IFNAR1, Leinco Technologies, St. Louis, Missouri, USA) was diluted in sterile H_20 to a stock concentration of 2.3mg/ml and stored in aliquots at -80°C. Cells were pre-treated with 2.5mg/ml MAR1 or IgG isotype control in appropriate culture media for 30 minutes prior to rotenone treatment. Cell viability of neurons was then determined by MTT assay after 24h or the media from glia removed after 24, 48 or 72h to measure type-I IFN levels by reporter assay.

For conditioned media experiments, wildtype or IFNAR1$^{-/-}$ primary glia were treated with 25nM rotenone (or vehicle) for 24, 48 or 72h or alternatively, wildtype glia only were treated with rotenone (25nM) in the presence of MAR-1 (2.5μg) or IgG control for 24h. Media was then removed and cell viability was assessed by MTT assay with values of rotenone-treated cells expressed as a percentage of their vehicle control.

MTT cell viability assay

Cell viability was measured by the ability of cells to metabolise 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reagent (2mg/ml, Sigma) to a formazan product. Following rotenone treatment, MTT was added to culture and incubated for 2h at 37°C/5% CO_2. Culture medium was then removed and 200μl DMSO was added to each well to solubilise the formazan product, followed by transferral to a 96 well plate and absorbance was determined at 595nm. All experiments were performed in triplicate and viability was expressed as a percentage of Abs_{595nm} of the vehicle control.

RNA extraction and cDNA synthesis

Cells were lysed and homogenised in 1mL of Trizol® reagent (Invitrogen). RNA was isolated as per manufacturers guidelines, with concentration and purity of the RNA assessed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific). RNA was reverse transcribed into cDNA using a High-Capacity RNA-to-cDNA Reverse Transcription Kit (Applied Biosystems) in accordance with manufactures guidelines. Briefly, reverse transcription of 1μg RNA to cDNA was performed using thermocycler (Eppendorf) under the following conditions: 25°C/10min, 37°C/120min, 95°C/5min, 10min/4°C. The resulting cDNA was diluted 1:3 with DEPC-H_2O and stored at -80°C for QPCR analysis.

Quantitative Real Time Polymerase Chain Reaction (QPCR)

All QPCR was performed in standard 384-well plates (4309849, Applied Biosystems) using the 7900ht fast real-time PCR system (Applied Biosystems) and reactions for a given sample were performed in triplicate. All Taqman gene expression assays were purchased commercially (431182, Applied Biosystems) and reactions were performed under the following conditions with the listed primers:

Step #	Temperature (°C)	Time (minutes)	Comments
1	50	2	-
2	94.5	10	-
3	97	0.5	X40 repeats
4	59.7	1	

Gene	Species	Inventory number
GAPDH	Mouse	Mm99999915_m1
IFNβ	Mouse	Mm00439552_s1
IL-1β	Mouse	Mm01336189_m1
TNFα	Mouse	Mm00443258_m1

For SYBR® green-based detection, gene-specific primers were synthesized commercially (Geneworks) and reactions were performed under the following thermal conditions:

Step #	Temperature (°C)	Time (minutes)	Comments
1	95	20	-
2	95	0.5	X40 repeats
3	60	1.5	
4	95	15	-
5	60	15	-
6	95	15	-

Gene	Direction (5’ → 3’)	Sequence (5’→ 3’)
GAPDH	Forward	ATCTTCTTGTGCAGTGCCAGC

	Reverse	ACTCCACGACATACTCAGCACC
IFNα	Forward	GCAATCCTCCTAGACTCACTTCTGCA
	Reverse	TATAGTTCCTCACAGCCAGCAG
IFNαE4	Reverse	TATTTCTTCATAGCCAGCTG
iNOS	Forward	CAAGCACCTTGGAAGAGGAG
	Reverse	AAGGCCAAACACAGCATACC
CD16	Forward	TTTGGACACCCAGATGTTTCAG
	Reverse	GTCTTCCTTGAGCACCTGGATC
CD11b	Forward	CCAAGACGATCTCAGCATCA
	Reverse	TTCTGGCTTGCTGAATCCTT
CD86	Forward	TTGTGTGTGTTCTGGAAACGGAG
	Reverse	AACTTAGAGGCTGTGTTGCTGGG
ARG1	Forward	TCACCTGAGCTTTGATGTCG
	Reverse	CTGAAAGGAGCCCTGTCTTG
YM1	Forward	CAGGGTAATGAGTGGGTTGG
	Reverse	CACGGCACCTCCTAAATTGT

The software SDS 2.4 (Applied Biosciences) was used to generate threshold cycle (Ct) values. Ct values were collected and relative gene transcript levels were calculated using the δδCT method as described in (Winer *et al.* 1999).

Caspase-3 activity assay

Caspase-3 activity was measured by quantitating the ability of cells to cleave Ac-DEVD-pNa into free colorimetric pNA using the colorimetric CaspACE™ assay system (Promega, G7220) as per manufacturer's guidelines. Briefly, 50µg/well of protein extract (as determined by Bradford assay (Bio-rad)) was loaded in triplicate in a 96 well plate, and incubated at 37°C for 4h. Absorbance was read at 405nM and levels of cleaved pNa released for each sample was calculated relative to a standard pNa substrate curve.

B16-Blue™ type-I IFN reporter assay

B16-Blue™ IFNα/β reporter cells (bb-ifnt1, Gibco/Invitrogen, Scoresby, VIC, Australia) were used to measure type-I IFNs produced by primary cultured glial cell lines. B16-Blue™ murine type-I IFN reporter cells were derived from a murine B16 melanoma cell line of C57BL/6 origin. These cells have been stably transfected with a secreted embryonic

alkaline phosphatase (SEAP) reporter gene under the control of the IFNα/β-inducible ISG54 promoter enhanced by a multimeric Interferon Stimulated Response Elements (ISRE). Stimulation of these cells with murine IFNα/β triggers the production of SEAP by the activation of the IRF-inducible promoter. Levels of SEAP in the supernatant are then determined with QUANTI-Blue™ (rep-qb1, Gibco/Invitrogen, Scoresby, VIC, Australia), a colorimetric enzyme assay developed to determine any alkaline phosphatase (AP) activity in a biological sample. The colour of QUANTI-Blue™ changes from pink to purple/blue and absorbance determined at 630nm using a Multiskan Ascent plate reader (ThermoScientific, Scoresby, VIC, Australia).

Following treatments, conditioned media was collected and added to the B16-Blue™ reporter cells (bb-ifnt1, Invivogen, CA, USA) for 20-24 h. To validate reporter activity, B16-Blue™ reporter cells were concurrently treated with IFN-β (0-1000 IU/ml, PBL Assay Science, Piscataway, NJ, USA) to generate a standard curve for normalisation of results. QUANTI-Blue™ was added in a 1:1 v/v ratio to detect SEAP activity and incubated for 24h at 37°C/5% CO_2. Following SEAP detection, 200µl was transferred into a 96 well plate and absorbance determined at 630nm using Multiskan Ascent plate reader.

ELISA

Murine IL-6, IL-1β and IL-10 (BD Biosciences) ELISAs were performed according to manufacturer's instructions. 100µg of protein was loaded per well in duplicate. Protein concentrations of individual samples were determined using a linear curve of mouse IL-6, IL-1β or IL-10 standards (4-250pg/mL).

Statistical analysis

All data are expressed as mean ±SEM, n = number of experimental replicates. QPCR, ELISA and MTT assay data were analysed using a two-way ANOVA, with subsequent Bonferroni *post-hoc* tests. Power values for each test were calculated *post-hoc* using G*Power (version 3.1, http://gpower.hhu.de/), based upon the effect size, group number and sample size. For all statistical tests $P<0.05$ was accepted as being statistically significant.

Results

IFNAR1$^{-/-}$ primary cultured neurons are less sensitive to rotenone-induced toxicity

We have previously confirmed that IFNAR1$^{-/-}$ mice are less susceptible to MPTP-induced DA cell death *in vivo* (Main et al. 2016). To confirm that reduced type-I IFN signaling was neuroprotective *in vitro*, wildtype and IFNAR1$^{-/-}$ neurons were treated with rotenone (1-5nM) for 24h and cell viability was assessed by MTT assay. IFNAR1$^{-/-}$ neurons were less susceptible to cell death induced by 1nM (WT: 74.7±6.2% versus IFNAR1$^{-/-}$: 94.9±3.5% compared to vehicle, n=5, ***p<0.001, t=4.176, DF=3) and 3nM (WT: 75.1±2.8% versus IFNAR1$^{-/-}$: 93.3±2.8% n=4, **p<0.01, t=3.588, DF=3) rotenone compared to wildtype cultures (Figure 1A). It is well established that rotenone can induce neuronal death via caspase-3 mediated apoptosis (Ahmadi *et al.* 2003, Pei & Pei 2003). To investigate whether the neuroprotection we observed in IFNAR1 deficient neurons was due to reduced caspase-3 activity, cells were treated with 3nM rotenone for 8, 24 and 48h and levels of pNA measured. IFNAR1$^{-/-}$ primary cultured neurons displayed a significant decrease in caspase-3 activity at 24h (WT: 127.0±19.0pmol/50μg vs IFNAR1$^{-/-}$: 62.2±5.0pmol/50μg, n=6, *p<0.05, t=2.622, DF=3) and 48h (WT: 134.7±22.2pmol/50μg vs IFNAR1$^{-/-}$: 83.0±6.4pmol/50μg, n=6, *p<0.05, t=2.847, DF=3) compared to wildtype neurons (Figure 1B).

To correlate our *in vitro* viability studies with a reduced pro-inflammatory response (including the type-I IFNs) in the IFNAR1$^{-/-}$ neurons, cells were treated with rotenone over a 2-48h time-course. QPCR analysis was performed to determine expression of both the type-I IFNs and the classical pro-inflammatory cytokines, IL-1β and TNF-α. Rotenone induced a significant increase in IFN-α expression in wildtype neurons at 2h (WT: 8.5±1.1-fold versus IFNAR1$^{-/-}$: 1.4±0.4-fold compared to vehicle, n=5, t=4.053, ***p<0.001, DF=5) and 4h (WT: 6.5±1.8-fold versus IFNAR1$^{-/-}$: 0.8±0.2-fold compared to vehicle, n=6, **p<0.01, t=3.653, DF=5) that was absent in the IFNAR1$^{-/-}$ neurons (Figure 2A). Similarly, there was a significant difference in the levels of IFN-β between wildtype and IFNAR1$^{-/-}$ neurons at 4h (WT: 4.4±1.0-fold versus IFNAR1$^{-/-}$: 1.2±0.2-fold compared to vehicle, n=6, **p<0.01, t=3.272, DF=5) (Figure 2B). At 48 hours, there was a second wave of increased IFN-α (WT: 8.0±1.6-fold versus IFNAR1$^{-/-}$: 3.0±1.0-fold compared to vehicle, **p<0.01, n=8, t=3.588, DF=5) and IFN-β (WT: 5.3±0.9-fold versus IFNAR1$^{-/-}$: 2.3±0.9-fold compared to vehicle, n=7, **p<0.01, t=3.408, DF=5) expression in the rotenone-treated wildtype neurons that was attenuated in the IFNAR1$^{-/-}$ cells. Over the entire rotenone time-course, the knockout neurons also displayed reduced expression of IL1-β and TNF-α, with significant differences at 24h (WT: 2.3±0.4-fold versus IFNAR1$^{-/-}$: 0.6±0.1-fold compared to vehicle, n=6 *p<0.05,

t=2.957, DF=4) and 48h (WT: 2.7±1.2-fold versus IFNAR1$^{-/-}$: 0.7±0.1-fold compared to vehicle, n=7 **p<0.01, t=3.529, DF=4) and 4h (WT: 4.1±1.2-fold versus IFNAR1$^{-/-}$: 1.4±0.4-fold compared to vehicle, n=6, *p<0.05, t=3.126, DF=4), respectively (Figure 2C, D).

IFNAR1$^{-/-}$ glia display an attenuated pro-inflammatory response induced by rotenone

Glial cells (microglia and astrocytes) are considered the predominant cell type involved in the neuroinflammatory response in both acute and chronic brain neuropathologies. We were interested in identifying whether type-I IFN signaling was also critical in mediating the glial response to rotenone. Once it was confirmed rotenone was not toxic to the primary mixed glial cultures at the doses of 1-100nM (data not shown), a time course of 24-72h with 25nM rotenone was performed. Expression of IFN-α and IFN-β was determined by QPCR analysis with a type-I IFN reporter cell line used to measure type-I IFN levels released into the glial media. At 24h, rotenone induced an increase in type-I IFN expression in wildtype glia with mRNA levels of IFN-α significantly elevated compared to IFNAR1$^{-/-}$ cells (WT: 5.3±1.5-fold versus IFNAR1$^{-/-}$: 1.5±0.4-fold compared to vehicle, n=6, *p<0.05, t=2.652, DF=3) (Figure 3A). Similarly, at 72h there was an attenuated response in the knockout cells with reduced expression of both IFN-α (WT: 7.3±1.3-fold versus IFNAR1$^{-/-}$: 0.5±0.1-fold compared to vehicle, n=6, **p<0.01, t=3.670, DF=3) and IFN-β (WT: 7.3±2.0-fold versus IFNAR1$^{-/-}$: 1.2±0.5-fold compared to vehicle, n=6, *p<0.05, t=2.821, DF=3) (Figure 3A, B). In support of the reduced mRNA expression, IFNAR1$^{-/-}$ glia demonstrated significantly reduced type-I IFN protein levels at both 24h (WT: 37.9±6.9 IU/mL versus IFNAR1$^{-/-}$: 6.2±2.5 IU/mL, n=4, ***p<0.01, t=3.988, DF=3) and 72h (WT: 79.8±8.2 IU/mL versus IFNAR1$^{-/-}$: 40.7±13.9 IU/mL, n=4, ***p<0.001, t=4.157, DF=3) (Figure 3C). Analysis of pro-inflammatory cytokine expression by ELISA confirmed a significant increase in IL-6 levels at 24h (WT: 200.7±186.7 pg/200μL versus IFNAR1$^{-/-}$: 34.3±9.0 pg/200μL, n=6, **p<0.01, t=3.245, DF=3) (Figure 3D) and IL1-β levels at 48h (WT: 121.3±26.7 pg/200μL versus IFNAR1$^{-/-}$: 58.6±9.9 pg/200μL, n=6, **p<0.05, t=3.245, DF=3) (Figure 3E) in the media from rotenone-treated wildtype, but not IFNAR1$^{-/-}$ glia. In contrast, rotenone only upregulated IL-10 expression levels in the IFNAR1$^{-/-}$ cells at 24h (WT: 35.1±9.8 pg/200μL versus IFNAR1$^{-/-}$ -: 146.3±57.2 pg/200μL, **p<0.01, t=3.273, DF=3) (Figure 3F). This suggests that type-I IFN signaling is a critical mediator of the pro-inflammatory response induced by rotenone in primary glia.

Wildtype, but not IFNAR1$^{-/-}$ glia, display increased expression of M1-like markers in response to rotenone

Studies of the innate immune response have highlighted its complexity with a myriad of factors, their receptors and subsequent signaling pathways involved. Akin to their peripheral counterparts, in an attempt to simplify the response, the terms "M1" and "M2" have been widely used to designate pro- and anti-inflammatory microglia (and to a lesser extent astrocytes), respectively (reviewed in (Cherry *et al.* 2014)). However, the use of this "M1" of "M2" nomenclature is now considered an oversimplification, with evidence demonstrating that microglia exist as a continuum of the two or as a mixed phenotype in response to specific stimuli (reviewed in (Moehle & West 2015)). The increased pro-inflammatory cytokine expression induced by rotenone in wildtype mixed glia (Figure 3D,E) did suggest a propensity of the cultures to adopt a more "M1-like" phenotype overall. However to confirm this, mRNA expression of M1 (CD11b, CD16, CD86 and iNOS) and M2 (ARG1 and YM1) markers was analysed by QPCR. Expression of CD11b (WT: 2.9±0.7-fold versus IFNAR1$^{-/-}$: 0.7±0.1-fold compared to vehicle, n=6, ***p<0.001, t=5.260, DF=3), CD16 (WT: 2.3±0.4-fold versus IFNAR1$^{-/-}$: 0.9±0.1-fold compared to vehicle, n=6, ***p<0.001, t=4.380, DF=3) and CD86 (WT: 2.8±1.2-fold versus IFNAR1$^{-/-}$: 1.0±0.1-fold compared to vehicle, n=6, *p<0.05, t=2.922, DF=3) were all significantly upregulated in wildtype but not IFNAR1$^{-/-}$ cultures following 72h rotenone treatment (Figure 4A-C). Rotenone also induced an early increase in iNOS expression at 24h in wildtype glia, a response that was attenuated in the knockout cultures (WT: 2.1±0.5-fold versus IFNAR1$^{-/-}$: 1.0±0.1-fold compared to vehicle, n=3, *p<0.05, t=2.862, DF=3) (Figure 4D). In contrast, there was no significant difference in ARG1 and YM1 expression between the wildtype and IFNAR1$^{-/-}$ cells with an upregulation in both genotypes. (Figure 4E, F). This data suggests although wildtype glial cells do adopt a mixed phenotype exhibiting both pro- and anti-inflammatory responses, these cultures are over-represented by the former. The IFNAR1$^{-/-}$ cells in contrast, with little evidence of increased M1 marker expression by rotenone, display a more anti-inflammatory phenotype as a whole. The elevated levels of IL-10 accompanied by minimal increases in IL-6 or IL-1β in these cultures supports this (Figure 3D-F).

Conditioned media from rotenone-treated IFNAR1$^{-/-}$ glia is less cytotoxic to primary cultured neurons

Wildtype or IFNAR1$^{-/-}$ primary glia were treated with 25nM rotenone for 24, 48 or 72h before the media was removed and placed onto primary cultured wildtype neurons for 24h and cell viability determined by MTT assay. Increased cell death was seen in wildtype neurons that were exposed to media from rotenone-treated (24h) wildtype glia compared to IFNAR1$^{-/-}$ glia (78.4±2.4% versus 92.2±2.2% compared to vehicle, n=7, *p<0.05, t=3.148, DF=3, two-way ANOVA) (Figure 5). A significant difference in viability was also seen with media from glia treated with 25nM rotenone for 48h (WT: 73.41±3.9% versus IFNAR1$^{-/-}$: 84.7±3.8% compared to vehicle, n=7, *p<0.05, t=2.640, DF=3, two-way ANOVA, Bonferroni's *post hoc* test). The reduced cytotoxicity of media from IFNAR1$^{-/-}$ glia supports the previous data identifying reduced pro-inflammatory cytokine expression as a result of attenuated type-I IFN signaling.

MAR-1, an IFNAR1 blocking monoclonal antibody, is neuroprotective against rotenone-induced cell death

To further characterise the neuroprotective effects of blocking type-I IFN signalling, wildtype primary neurons were incubated with a blocking monoclonal IFNAR1 antibody, MAR-1 (2.5mg/ml) or IgG isotype control for 30 minutes before being treated with 3nM rotenone for 24h and cell viability assessed by MTT assay. In the presence of the IgG control antibody, rotenone-treated neurons demonstrated 75.6±2.9% viability (compared to IgG/vehicle control) (Figure 6A). In contrast, there was a significant attenuation of rotenone-induced cell death in the MAR-1 treated cells (90.4±5.2% compared to MAR-1/vehicle control) (n=3, *p<0.05, t=3.495, DF=3, one-way ANOVA, Bonferroni's *post hoc* test). MAR-1 was able to effectively block the rotenone-induced type-I IFN production in primary cultured glia (Figure 6B) as measured by reporter assay with a significant reduction at 72h (IgG/rotenone: 38.9±8.9IU/mL compared to IgG/vehicle versus to MAR-1/rotenone 13.2±1.9IU/mL, n=3, **p<0.01, t=4.495, DF=3, two-way ANOVA, Bonferroni's *post hoc* test). In addition, media from glia treated with rotenone in the presence of MAR-1 was significantly less cytotoxic to wildtype neurons compared to media from IgG/rotenone treated glia (IgG/rotenone: 70.2±6.3% cell viability compared to MAR-1/rotenone: 87.9±4.3% cell viability (relative to vehicle controls) (n=3, *p<0.05, t=3.277, DF=3, two-way ANOVA, Bonferroni's *post hoc* test). This data supports the neuroprotective effects seen with MAR-1 *in vivo* and suggests that this is in part due to its ability to attenuate the glial type-I IFN production to these toxins.

Discussion

Evidence suggests that neuroinflammation is a fundamental process that although beneficial following acute neural injury, contributes to the neurodegeneration in chronic neuropathologies. However, the key mediator of this inflammatory response and the mechanism of action that can lead to cell death remain undefined. Type-I IFNs possess pleiotropic biological functions including anti-viral and anti-proliferative capacities and are considered to be the key initiator of the inflammatory response. Type-I IFN-IFNAR signaling and subsequent activation through the classical JAK/STAT cascade leads to an upregulation in pro-inflammatory gene transcription including the type-I IFNs themselves (de Weerd *et al.* 2012). This signaling cascade has been well characterised in the periphery, however its contribution to the neuroinflammatory response in chronic CNS disorders such as AD and PD is still not known. Our previous data implicated the type-I IFNs in the MPTP model of PD (Main et al. 2016) and in the APP/PS1 mouse model of AD (Minter et al. 2016a) with an attenuated neuroinflammatory response in mice lacking IFNAR1. We hypothesised that glial cells are the major drivers of this damaging response, however as both neurons and glia are known to produce and respond to the type-I IFNs, this study further characterised type-I IFN signaling in specific CNS cell types following exposure to the neurotoxin rotenone. Rotenone is a complex I inhibitor known to induce oxidative stress and neuronal cell death but recently it has also been reported to activate microglia with subsequent pro-inflammatory cytokine secretion (Ye et al. 2016). We confirmed that rotenone induces a pro-inflammatory response that includes increased type-I IFN expression, in both primary cultured neurons and glia. Removal of the type-I IFN receptor, IFNAR1, in these cells attenuated this pro-inflammatory response with subsequent neuroprotection against rotenone-induced neuronal cell death. Significantly, by therapeutically targeting IFNAR1 through the monoclonal antibody, MAR-1, we were also able to provide some protection against cell death induced by rotenone.

The resident microglia are considered the primary immune cell in the CNS and therefore have been implicated as the major driver of neuroinflammation in both AD and PD (reviewed in (Taylor *et al.* 2013, Minter *et al.* 2016b)). However, neurons and astrocytes are also involved in this response and it is known that neurons also express and respond to type-I IFNs through TLR stimulation (Costello *et al.* 2013, Carpentier *et al.* 2005). In this study, we demonstrate that rotenone treatment stimulates an early up-regulation (within 2h) in type-I

IFN expression in wild type primary neuronal cultures. Furthermore this type-I IFN production occurs before the up-regulation of pro-inflammatory cytokines (IL1-β and TNF-α) supporting a role for the type-I IFNs as mediators or regulators of the neuroinflammatory environment induced by rotenone. Similarly, we have previously reported that the Aβ1–42-induced IFN response in primary neurons occurs prior to the induction of other pro-inflammatory cytokines (Taylor *et al.* 2014). This has implications for the human conditions where hallmark features of neuroinflammation including the expression of IL-1β and TNF-α have been detected in both the CSF from AD and PD patients and in post-mortem brains. Additionally, studies have demonstrated that neurons are sensitive to IL-1β and TNF-α cytotoxicity (Kaur et al 2012). Attenuation of this pro-inflammatory response through TNF-$\alpha^{-/-}$ mice (Ferger *et al.* 2004), soluble TNF-α inhibitors (McCoy & McCoy 2006) and anti-IL-1β neutralising antibodies (Arai *et al.* 2006) are neuroprotective, further demonstrating the role of the inflammatory response in disease progression. Similarly, our data reveals that primary cultured neurons from IFNAR1$^{-/-}$ mice not only have attenuated type-I IFN expression, but also reduced rotenone-induced pro-inflammatory cytokine production of TNF-α and IL-1β compared to wildtype controls. This reduced response in the IFNAR1$^{-/-}$ neurons correlated with decreased rotenone-induced cell death compared to wild type cultures that was associated with reduced caspase-3 activity. In support of this, BE(2) M17 neuroblastoma cells stably overexpressing a shRNA IFNAR1 knockdown construct also displayed reduced rotenone-induced neuroinflammation and cell death (data not shown) whilst we have previously confirmed an attenuated neuroinflammatory response in IFNAR1$^{-/-}$ neurons with subsequent protection against Aβ1-42-induced cell death (Taylor *et al*. 2014). Indeed, IFN-β has been shown to have deleterious effects on neuronal survival, by regulating signaling molecules that promote intrinsic apoptosis (Dedoni *et al.* 2010). These results suggest a key modulatory role for type-I IFNs in not only the pro-inflammatory response but also in the downstream pro-apoptotic signaling mechanisms induced by rotenone and other cellular insults. In addition, it supports our previous findings in the MPTP mouse model of PD that identified increased DA neuronal cell survival in the absence of IFNAR1 (Main *et al.* 2016).

Rotenone was also shown to be a potent stimulator of the type-I IFNs in primary glial cells. Previous studies have confirmed a pro-inflammatory response in rotenone-treated mixed primary cultured glia or BV2 microglia cells with increased expression of IL1-β and TNF-α reported (Liang *et al.* 2015, Chang *et al.* 2013). Type-I IFN levels were upregulated

within 24h and in contrast to the IL-6 and IL-1β levels which returned to near control levels at 72h, there was a second "wave" of IFN production at the latter time-point. This could be attributed to secondary signaling pathways involving damage-associated molecular patterns (DAMPs) leading to elevated type-I IFNs (Gao *et al.* 2011) and mimics what we also saw in glial cells in response to Aβ1-42 (Minter *et al*. 2016a). Again, highlighting the modulatory role of the IFN-IFNAR1 signaling cascade, this pro-inflammatory cytokine expression was diminished in the IFNAR1$^{-/-}$ glia. In contrast, expression of the anti-inflammatory IL-10 was only upregulated in the IFNAR1$^{-/-}$ glia. This imbalance in the WT glia only (and not in the IFNAR1$^{-/-}$ cells) was further corroborated by the mRNA expression of M1-microglial markers. Whilst "M2" markers were unchanged in either rotenone-treated WT or IFNAR1$^{-/-}$ mixed glia cultures, the expression of "M1" markers, CD11b, CD16, CD86 and iNOS were all elevated in the WT (but not IFNAR1$^{-/-}$) cultures. We therefore concluded similar to our *in vivo* MPTP study, rotenone induces a more M1-like phenotype in glia that is associated with the classical pro-inflammatory response as previously reported (Du *et al.* 2014). Interestingly, this contrasted to the upregulation in "M2" marker expression that we previously saw in Aβ1-42-treated IFNAR1$^{-/-}$ glia (and in APP/PS1/IFNAR1$^{-/-}$ mice), suggesting in the absence of type-I IFN signaling, these cells were adopting an anti-inflammatory phenotype (Minter *et al*. 2016a). However, taken together these studies do suggest that the type-I IFNs drive or influence glia to adopt a pro-inflammatory phenotype. Indeed, the detrimental effects of this increased pro-inflammatory cytokine secretion was confirmed when the media from the rotenone-treated wildtype glia was placed onto primary cultured neurons. The reduced neurotoxicity in the absence of a glial mediated IFN response is similar to that reported with the type-2 IFN, IFNγ (Mount *et al.* 2007) and indeed with our own studies with Aβ-treated neurons/glia (Minter *et al*. 2016a).

We have previously confirmed that blocking the type-I IFN receptor through the use of a monoclonal antibody, is effective in reducing type-I IFN expression in the brain, pro-inflammatory cytokine expression and ultimately nigral degeneration induced by MPTP (Main et al. 2016). This study has confirmed that rotenone induces type-I IFNs in both neurons and glia and critically, abrogation of this response through MAR-1 treatment, afforded some protection against subsequent neuronal cell death. Identifying the cellular source of the type-I IFNs and confirming the implications of targeting this response were critical to confirm this pathway as a true therapeutic target for future drug design. We do not propose that type-I IFN signaling causes AD or PD, however we hypothesise that the type-I

IFNs are critical mediators of the neuroinflammatory response that contributes to the neuronal degeneration in these diseases. Our studies support the notion that therapeutically targeting this response, particularly that occurring in the glial cells of the CNS, may provide an alternative strategy to reduce neurodegeneration and slow the progression of these debilitating diseases.

ARRIVE guidelines have been followed:
Yes
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Acknowledgments/Conflicts of interest disclosure

This work was supported by a University of Melbourne Early Career Fellowship (JMT) and an Australian Rotary Health Postgraduate Scholarship (BSM). JMT and PJC are funded through the National Health & Medical Research Council (Australia). PJC is a recipient of an Australian Research Council (ARC) Future Fellowship. The authors have no conflict of interests to declare.

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Figure Legends

Fig. 1. IFNAR1$^{-/-}$ primary neurons display reduced caspase-3 mediated rotenone-induced cell death. (A) Primary cultured wildtype (WT) and IFNAR1$^{-/-}$ neurons were treated with 1, 3 or 5nM rotenone or vehicle for 24h before cell viability was assessed by

MTT assay (n=4-5). All data expressed relative to vehicle control. **(B)** WT and IFNAR1$^{-/-}$ neurons (n=6) were treated with 3nM rotenone for 8, 24 or 48h. Cells were harvested and caspase-3 levels assessed by measuring pNA levels as determined by referencing to a known pNA standard curve. Data expressed as the mean±SEM. Statistical analysis done by two-way ANOVA with Bonferroni's multiple comparison test (*p<0.05, **p<0.01, ***p<0.001).

Fig. 2. Rotenone treated IFNAR1$^{-/-}$ neurons display attenuated type-I IFN and pro-inflammatory cytokines expression. Primary cultured wildtype (WT) and IFNAR1$^{-/-}$ neurons were treated with 3nM rotenone or vehicle for 2-48h before cells were harvested and mRNA expression of **(A)** IFN-α **(B)** IFN-β **(C)** IL-1β and **(D)** TNF-α was determined by QPCR. All data expressed relative to vehicle control as the mean±SEM, n=6-8. Statistical analysis done by two-way ANOVA with Bonferroni's multiple comparison test (*p<0.05, **p<0.01, ***p<0.001).

Fig. 3. Rotenone induces a type-I IFN and pro-inflammatory response in wildtype glia that is diminished in IFNAR1$^{-/-}$ cells. Primary cultured wildtype (WT) and IFNAR1$^{-/-}$ mixed glia were treated with 25nM rotenone or vehicle for 24-72h before cells were harvested and mRNA expression of **(A)** IFN-α **(B)** IFN-β levels was determined by QPCR. **(C)** Type-I IFN expression as measured by HEK reporter assay with levels determined by comparison to a known IFN-β standard curve. Levels of IL-1β **(D)**, TNF-α **(E)** and the anti-inflammatory IL-10 **(F)** were assessed by ELISA. All data expressed relative to vehicle control as the mean±SEM, n=6. Statistical analysis done by two-way ANOVA with Bonferroni's multiple comparison test (*p<0.05, **p<0.01, ***p<0.001).

Fig. 4. The expression of pro-inflammatory "M1-like" microglial markers is elevated in wildtype, but not IFNAR1$^{-/-}$ glia, following rotenone treatment. Primary cultured wildtype (WT) and IFNAR1$^{-/-}$ mixed glia were treated with 25nM rotenone or vehicle for 24-72h before cells were harvested and mRNA expression of M1 markers **(A)** CDllb **(B)** CD16 **(C)** CD86 and **(D)** iNOS levels was determined by QPCR. The mRNA expression of arginase-1 (ARG1) **(E)** and YM1 **(F)** was unchanged between wildtype and IFNAR1$^{-/-}$ glia. All data expressed relative to vehicle control as the mean±SEM, n=6. Statistical analysis done by two-way ANOVA with Bonferroni's multiple comparison test (*p<0.05, ***p<0.001).

Fig. 5. Media from rotenone-treated IFNAR1$^{-/-}$ glia is less neurotoxic than wildtype glia to wildtype neurons. Primary cultured wildtype (WT) and IFNAR1$^{-/-}$ mixed glia were treated with 25nM rotenone or vehicle for 24-72h before the media was removed an applied to primary wildtype neurons for an additional 24h. Cell viability was determined by MTT assay with data expressed relative to vehicle control as the mean±SEM, n=7. Statistical analysis done by two-way ANOVA with Bonferroni's multiple comparison test (*p<0.05)

Fig. 6. MAR-1 inhibits type-I IFN signaling and rotenone-induced neurotoxicity. (A) Primary cultured neurons were pre-treated with a blocking monoclonal IFNAR1 antibody, MAR-1 (2.5μg) or IgG control for 30 minutes before being treated with 3nM rotenone (or vehicle (V)) for 24 hours. Cell viability was determined by MTT assay and confirmed a significant attenuation of rotenone-induced cell death in MAR-1 treated cells compared to IgG control cells (n=3). **(B)** MAR-1 (2.5μg) was shown to attenuate the type-I IFN response induced by rotenone (25nM) in primary cultured glia as measured by a HEK reporter assay (n=3). **(C)** Neuronal cell death induced by conditioned media from rotenone-treated primary glia was reduced in the presence of MAR-1 (2.5μg) compared to IgG-treated cells. All data expressed relative to vehicle control and as the mean±SEM, *p<0.05, **p<0.01, ***p<0.001.

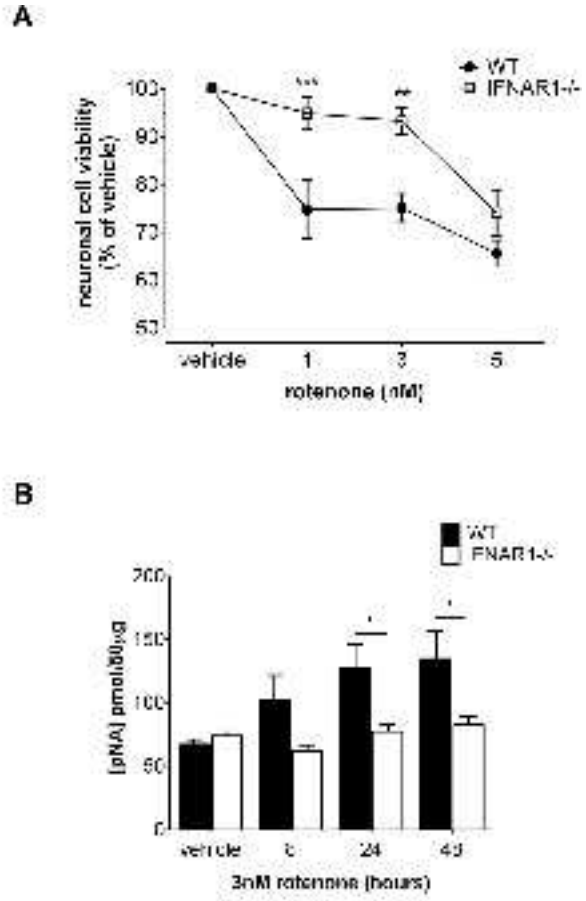

Figure 1

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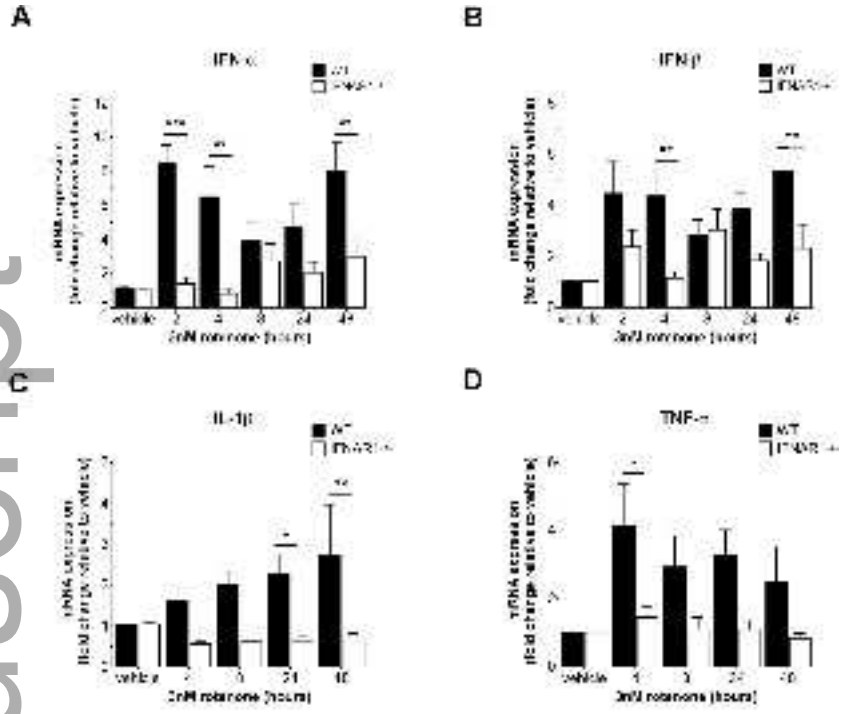

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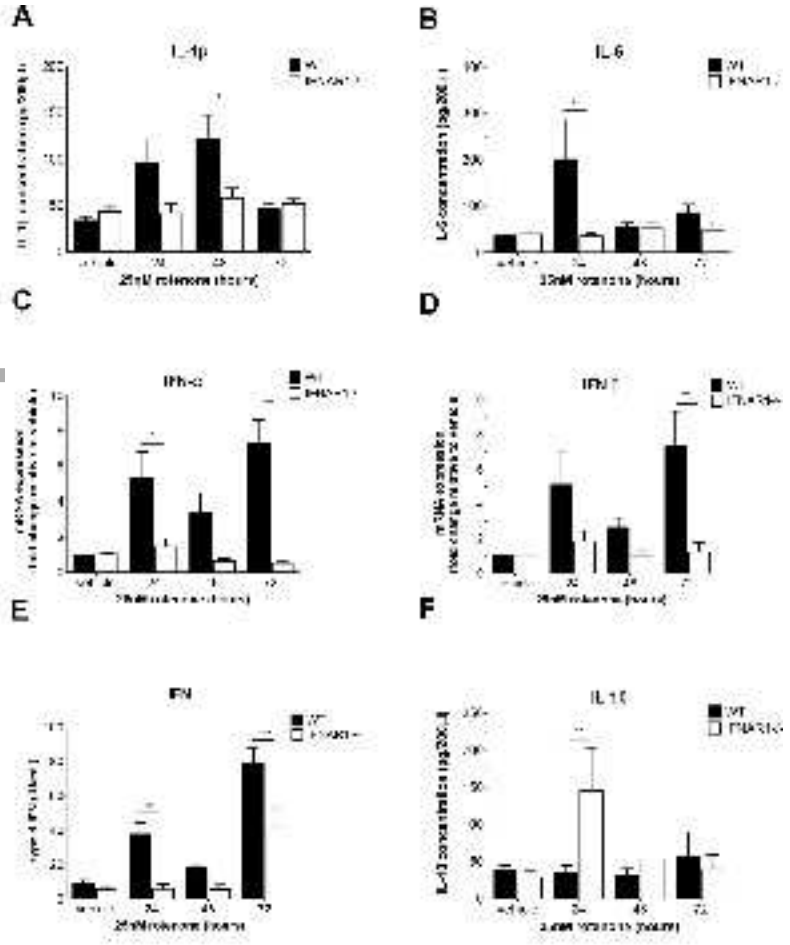

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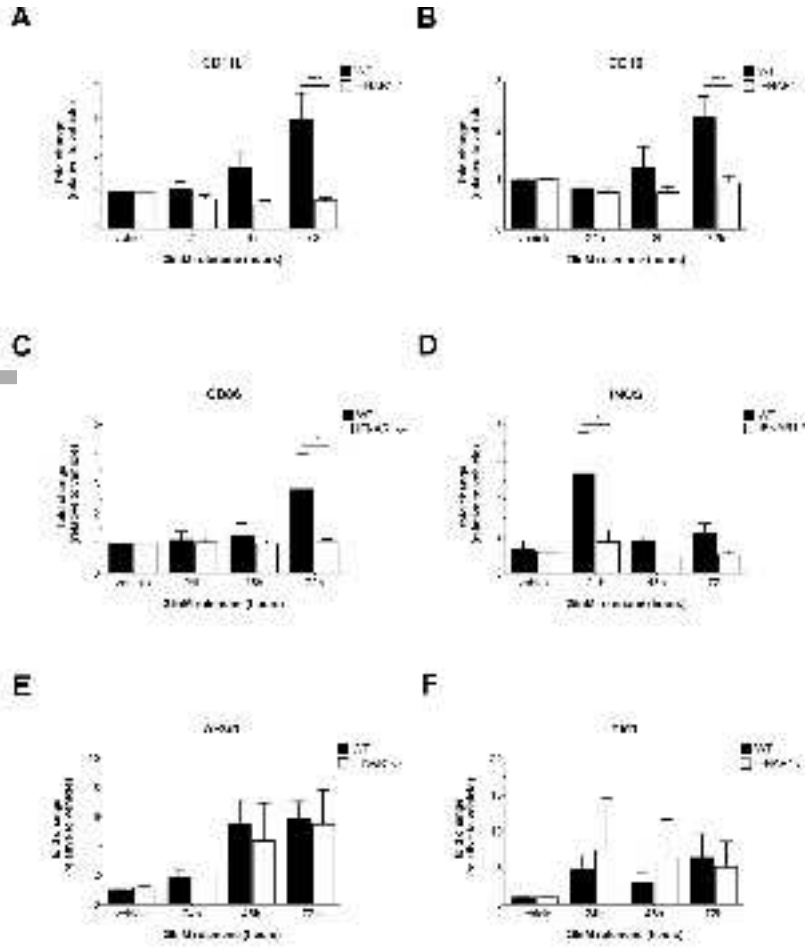

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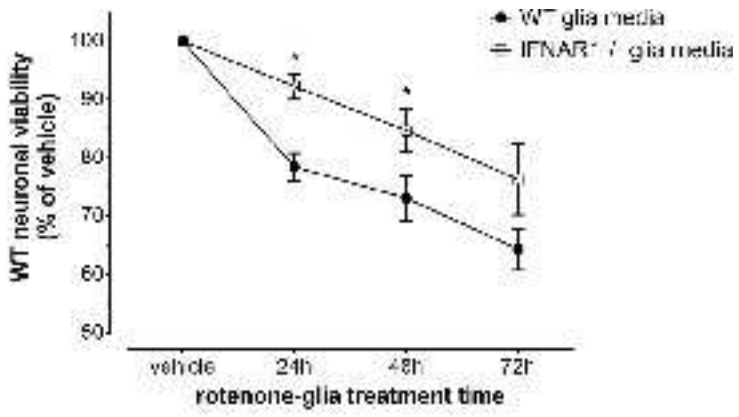

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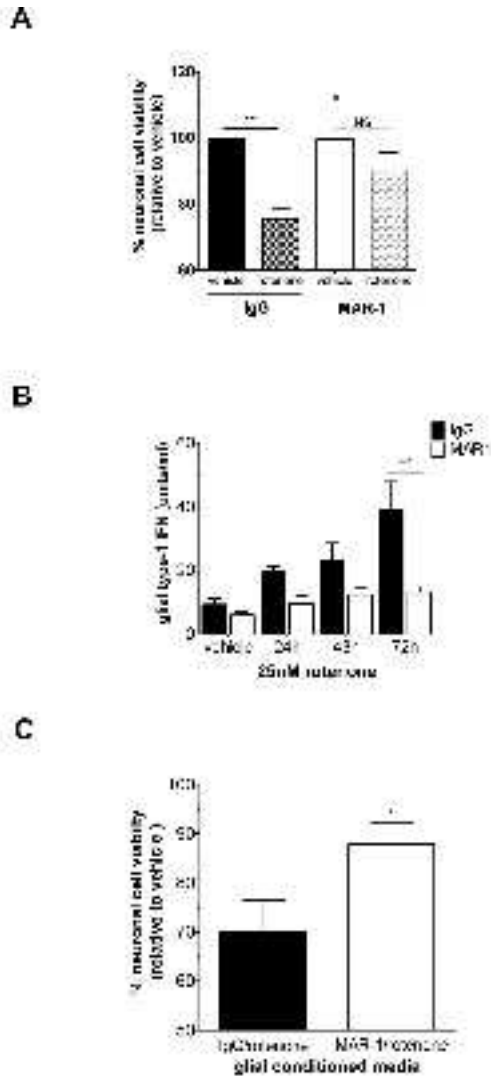

Figure 6

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