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Ifngr1 and Stat1 mediated canonical Ifn- γ signaling drives nigrostriatal degeneration

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Abstract

Brain expression of AAV-Ifn- γ leads to reactive gliosis, nigrostriatal degeneration and midbrain calcification in wild type mice. This mouse model phenocopies idiopathic basal ganglia calcification which is associated with Parkinsonian symptoms. To understand how the nigrostriatal pathway is selectively vulnerable to Ifn- γ , we determined if the phenotype is driven by canonical signaling intermediates, Ifngr1 and Stat1. Using focused bioinformatic analysis and rotarod testing, we show that neuroinflammation and motor abnormalities precede the appearance of midbrain neuropathologies in the brains of Ifn- γ mouse model. To test whether canonical Ifn- γ signaling is a key driver of progressive nigrostriatal degeneration, we overexpressed Ifn- γ in the brains of *Ifngr1*^{-/-} and *Stat1*^{-/-} mice. Overexpression of Ifn- γ in *Ifngr1*^{-/-} mice did not result in any neuroinflammation, midbrain calcinosis or nigrostriatal degenerative pathology. Interestingly, in *Stat1*^{-/-} mice, Ifn- γ expression resulted in gliosis without recapitulating the neurodegenerative phenotype. Overall, our data shows that canonical Ifn- γ signaling triggers midbrain calcinosis and nigrostriatal neurodegeneration, providing mechanistic insights into cytokine-driven selective neuronal vulnerability. Our study establishes the broader relevance of inflammatory signaling in neurodegenerative diseases and can potentially identify novel immunological targets for Parkinsonian syndromes.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Contains Supplementary Figures 1–4.

Author contribution

PC planned experiments, analyzed data and wrote the manuscript; PC injected neonatal mice; MRS, EJK, DZD and PC performed immunohistochemistry and analysis; DZD performed rotarod; CC-D and MRS performed NanoString and analyzed data; TEG provided overall support and critique.

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Graphical Abstract

Keywords

canonical Ifn- γ signaling; neurodegeneration; nigro-striatal pathway; neuroinflammation

Introduction

Neurodegenerative proteinopathies such as Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and Alzheimer's disease (AD) show different patterns of selective neurodegeneration. Yet, the proteins that are the presumed etiologic triggers of these diseases are typically widely expressed. Therefore, the differential vulnerability of subsets of neurons to various pathological factors remains an enigma [1–4]. Although numerous hypothesis have been proposed to account for differential neuronal vulnerability, there remains a poor understanding of why one population of cells is more vulnerable to a given neurodegenerative process than another. Understanding differential neuronal vulnerability may provide novel insights into neuroprotective strategies, and thus is of tremendous importance. The most simplistic conceptualization of differential neuronal vulnerability posits that the vulnerability of a given neuronal population is related to both the stressor and the intrinsic response of those neurons to that stressor [5]. Additional factors relating to the local microenvironment (including non-neuronal cells) may also modulate both the strength of the stressor and neuronal response to the stressor determining why one population of neurons is more vulnerable than another.

An invariant feature of neurodegenerative diseases is neuroinflammation, which reflects an underlying innate immune activation state thought to be altered in a defined temporal sequence preceding the onset of the neurodegenerative cascade [6, 7]. Immune activation has been proposed to be one of the extrinsic modulating factors that can directly trigger selective neuronal degeneration in these neurodegenerative diseases [5]. We have previously demonstrated that recombinant AAV (AAV) mediated expression of mouse Interferon (Ifn)- γ in wild type (WT) mice induces progressive and selective nigrostriatal degeneration and midbrain calcinosis [8]. This mouse model (AAV-Ifn- γ) phenocopies human idiopathic basal

ganglia calcification (IBGC) syndrome, which is a disorder typically characterized by neuropsychiatric impairment, progressive Parkinsonism and bilateral calcification of the basal ganglia and cerebellum [9]. The neurodegenerative pathology seen in the AAV-Ifn- γ mice appears highly selective for the thalamus and nigrostriatal pathways, whereas the hippocampus and cortex remains largely unaffected [8]. To our knowledge, Ifn- γ mouse model is the first model in which this degree of progressive and selective nigrostriatal degeneration is induced by expression of an endogenous factor. The phenotype cannot be attributed to general neuroinflammation; it is not observed with chronic brain inflammation induced in a similar manner by expression of another pro-inflammatory cytokines, Interleukin (IL)-6 [8]. In the AAV-Ifn- γ mice, there is a progressive evolution of phenotypic changes – midbrain calcinosis is observed at 3 months and frank nigro-striatal loss is observed at 5 months [8]. To enable us to gain mechanistic insights into age-progressive selective neurodegeneration induced by inflammatory signaling, here we used genetic tools to determine how inflammatory signaling drives selective and progressive neurodegeneration in the brains of AAV-Ifn- γ mice.

To understand how Ifn- γ modulates selective vulnerability of the nigrostriatal pathway, herein, we asked the question - is Ifn- γ induced selective nigrostriatal degeneration phenotype dependent on canonical Ifn- γ signaling? Using mice (*Ifngr*^{-/-} and *Stat1*^{-/-}) that are genetically deficient in Ifn- γ signaling [10, 11], we demonstrate here that Ifn- γ induced neuropathologies is a direct result of canonical Ifn- γ signaling. Absence of *Ifngr1* or *Stat1* completely blocked the development of Ifn- γ induced midbrain calcinosis phenotype and nigrostriatal neurodegeneration phenotype. This implies that the intrinsic response of canonical Ifn- γ signaling (strength or specificity) within nigrostriatal neurons determines the vulnerability of this population.

Materials and methods

Experimental Design

Litters of neonatal WT mice were randomly allocated for AAV injections (control or Ifn- γ). Following aging, these mice were tested on the rotarod by a blinded experimenter (i.e., cage cards of mice only had numbers as identification tags) and euthanized within 7 days.

Mice

Mice were housed in groups of 3–4 in conventional cages (29 × 18 × 13 cm) at 20–26°C on a daily 12-hour light-dark cycle with *ad libitum* access to food and water. All procedures received prior approval from the Institutional Animal Care and Use Committee of the University of Florida. WT B6/C3H mice (n=32 mice total, n=4–6 mice/age/treatment) were obtained from Envigo (Indianapolis, IN), *Ifngr*^{-/-} mice (129-*Ifngr*1tm1Agt/J) (n=10 mice/age/treatment) from Jackson Labs (Bar Harbor, ME) and *Stat1*^{-/-} mice (n=8 mice/age/treatment) from the laboratory of Joan Durbin, PhD (New York University School of Medicine). *Ifngr*^{-/-} and *Stat1*^{-/-} mice strains are maintained on the C57/B6 genetic background. WT mice were analyzed at indicated timepoints whereas *Ifngr*^{-/-} or *Stat1*^{-/-} were analyzed at 5 months of age.

rAAV injection in neonates

We have previously established various paradigms for AAV mediated gene expression in the neonatal mouse brain that enable us to control the transgene dosage and transduction profiles by altering the timing of AAV injection [12]. Injection of AAV-Ifn- γ , that encodes the Type II interferon, into WT mice on neonatal day P2 leads to mostly choroid limited transduction of the AAV [8], thus ensuring that the action of Ifn- γ in this model is largely non-cell autonomous. Wild type B6/C3H mice were injected bilaterally into the cerebral ventricles with 2 μ l of AAV (1×10^{10} vector genomes) on neonatal day P2 (36–48hrs after birth), as previously described, aged and assessed for rotarod performance and neuropathology.

Rotarod performance test

Mice were placed on a rotarod (IITC Life Sciences Inc., Series 8, CA, USA), which accelerated from 4 to 30 rpm over 5 minutes. This test was repeated four times every day for four consecutive days with a 10-minute rest interval between each trial. Tests for rotarod performance were conducted at 1, 3, 5 and 8 months of age. Total distance moved (in meters) before falling of the rotarod was averaged across trials and were presented as the average distance for each treatment. Data are plotted as mean $\pm$ S.E. and statistical comparisons conducted using Student's unpaired t-test.

Analysis of differential gene expression by NanoString

Total RNA extracted from mouse brain was purified using TRIzol and RNAqueous kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) and reverse transcribed using Superscript III (Invitrogen, Life Technologies, Carlsbad, CA, USA). Multiplex analysis of RNA was done using the nCounter GX mouse inflammation kit (NanoString Technologies, Seattle, WA, USA) and a custom Neurodegeneration kit [13] using 100 ng total RNA from mouse brains.

NanoString Normalization and Analysis

All analysis was done in R v3.3.3 [14]. NanoString data was normalized using the NanoStringNorm package [15] using the following parameters: 1) CodeCount (positive controls): mean; 2) Background (negative controls): mean; and 3) SampleContent (housekeeping): sum. Any samples greater than three standard deviations from the mean were removed. A log₂ transformation of the data was taken. Volcano plot was generated using the R NanoStringNorm package. Normalized sample data from the commercially available NanoString Mouse Inflammation GX array and a custom NanoString Neurodegeneration array [13] were combined for further analysis. Log fold change and p-value was compared between the 1 month, 3 month, and 5 month Ifn- γ treated mice and 5 month control wild-type mice. A heat map was created from this data and hierarchical clustering was used to sort groups and create dendograms visualizing the clustering using the R d3heatmap package [16]. PCA analysis was done using the R stats package with the prcomp function with center and scale set to true [14]. Principal components 1 and 2 were plotted for visualization and explain 0.4227 and 0.1900 proportion of the variance and have a standard deviation of 12.0754 and 8.0972 respectively. K-Means analysis was done with a k of 2, an nstart parameter of 25 and a maximum of 1000 iterations. Within cluster sum of

squares by cluster was 65.8%. PCA plot was based on code from the R ggbiplot package [17]. Functional annotation clustering and enrichment analysis was done using DAVID Bioinformatics Resources 6.8 [18].

Biochemical and histological analyses

Brains were rapidly dissected, transected sagittally along the midline and the left hemisphere flash frozen. The frozen brain tissue was subsequently extracted with RIPA buffer as described previously [8]. Protein samples were separated on Bis-Tris 4–12% XT gels (Bio-Rad, Hercules, CA, USA) and probed with the following antibodies: glial fibrillary acidic protein (GFAP, 1:500; Chemicon, Billerica, MA, USA), Tyrosine hydroxylase (TH, 1:1000; Millipore), DARPP32 (1:500; Cell Signaling, Danvers, MA) and β -actin (1:1000; Sigma-Aldrich). Protein bands were detected using the multiplex Li-Cor Odyssey Infrared Imaging system (Li-Cor Biosciences, Lincoln, NE, USA). Relative band intensity was quantified using ImageJ software (NIH, Bethesda, MD). RIPA lysates were used to measure Ifn- γ levels using OptiEIA reagents (Becton Dickinson, Franklin Lakes, NJ, USA) per manufacturer's instructions. ELISA results were analyzed using SoftMax Pro software (Molecular Devices, Sunnyvale, CA, USA). Data are presented as mean $\pm$ S.E. and statistical analysis performed using Student's unpaired t-test.

The remaining right hemispheres were fixed with 10% normal buffered formalin (Fisher Scientific, Hampton, NH) followed by paraffin embedding. Antigen retrieval was done by water steaming the paraffin embedded sections for all antibodies except Stat1 and phosphorylated Stat1 (pStat1) which required steaming in 1mM EDTA (pH8.0) containing 0.05% Tween 20. Slides were subsequently stained with the following primary antibodies overnight at 4°C: GFAP (1:1000; Cell Signaling), Iba-1 (1:1000; Wako, Japan), TH (1:1000; Millipore), DARPP32 (1:1000; Cell Signaling), Stat1 (1:800, Cell Signaling) and Tyr 701 pStat1 (1:500; Cell Signaling). Color development was done using Vector ImmPress reagents (Vector Laboratories, Burlingame, CA, USA). All slides were counterstained with hematoxylin (Sigma, MO). Immunostained slides were quantified (% area) using the Positive Pixel Count program (Aperio) that detects DAB staining. At least three sections per sample, 30 μ m apart, were analysed by a blinded observer.

Statistical analysis

We used unpaired 2-tailed Student's t-tests or 1-way ANOVA for statistical comparisons (unless otherwise stated). Graphical representation of data was done using Prism 6 (GraphPad Software, La Jolla, CA) and final images were created using Photoshop CS4 (Adobe Systems).

Results

Ifn- γ expression leads to widespread neuroinflammation and motor deficits in WT mice

Following neonatal delivery of AAV-Ifn- γ in WT mice brain, we serendipitously discovered that Ifn- γ induced neuroinflammation leads to an extremely selective neurodegenerative phenotype in the basal ganglia and nigrostriatal pathway while largely sparing the forebrain, indicating that Ifn- γ selectively targets a vulnerable neuronal population in these brain areas

[8]. This mouse model phenocopies IBGC syndrome, characterized by bilateral basal ganglia calcification and a variety of neurological and psychiatric abnormalities, most frequently Parkinsonian movement disorder [19].

To rigorously characterize whether the selective neurodegenerative phenotype can be explained on a facile level based on the relative distribution of Ifn- γ protein in the brain, we dissected the brains of 3 month old AAV-Ifn- γ and AAV-EGFP (Control) mice to assess Ifn- γ levels. We found that compared to control mice, the levels of Ifn- γ protein was elevated in all parts of the brain, reaching the highest levels in the forebrain (cortex and hippocampus), followed by midbrain, striatum and cerebellum, with no apparent selectivity in protein distribution (Supplementary Figure 1A). We confirmed that expression of Ifn- γ leads to fulminant and uniform astrogliosis (GFAP immunohistochemistry) and microgliosis (Iba-1 immunohistochemistry) in all areas of the brain examined, e.g., midbrain, and basal ganglia (striatum and thalamus) as well as forebrain (cortex) (Supplementary Figure 1B–C). This neuroinflammatory phenotype is evident as early as 1 month of age lasting through the lifetime of the animal (Supplementary Figure 1B–C). Next, using two different NanoString arrays (commercial mouse Inflammation array and a custom-designed Neurodegeneration array [13]), we performed focused transcriptomic analysis to enable molecular immunophenotyping of the Ifn- γ mice. A heat map and principal component analysis of the differentially expressed genes (DEG) show that Ifn- γ expressing wild type mice at different ages (1, 3 and 5 months of age) are clustered together compared to 5 month old control mice (Figure 1A–B). This indicates that the Ifn- γ induced inflammatory gene expression changes occur early and are relatively conserved between the young and older age groups analyzed. Ifn- γ expression results in induction of known components of the Ifn- γ signaling pathway, such as Zbp1, Ccl8 and members of the major histocompatibility complex [20] (Figure 1C). Functional annotation and pathway mapping of the enriched DEG data set shows that biological effects of Ifn- γ in the systemic inflammation is recapitulated in the brain following AAV-Ifn- γ expression (Figure 1D). For example, the physiological response observed during Ifn- γ priming of systemic immunity through the Jak/Stat pathways during pathogen infections is reflected in the DEG analysis of the Ifn- γ expressing brains (Figure 1D) [21]. We also confirmed increased immunoreactivity of Stat1 and phosphorylated Stat1 (Tyr 701) in Ifn- γ expressing 5 month cohort compared to control mice, indicating the engagement of Stat1-mediated signaling pathways in these mice (Supplementary Figure 2).

To ensure the reproducibility of our earlier dataset [8], we analyzed this independent cohort of AAV-Ifn- γ mice for neuropathological impairments. We observed that Ifn- γ -induced dystrophic calcinosis generally occurs around 3 months of age (Supplementary Figure 3A–B), followed by nigrostriatal degeneration which is evident at 5 months of age, indicated by loss of DARPP32 immunoreactivity in the dopaminergic (DA) neurons and nerve terminals (Supplementary Figure 3A–C). Though frank nigrostriatal degeneration is observed only at 5 months of age, the Ifn- γ expressing mice display motor abnormalities as early as 1 month of age, which then worsens progressively with age, as assessed by rotarod performance test (Figure 2). Overall, this provides confirmatory evidence that the neurodegenerative phenotype is faithfully recapitulated in an independent cohort and additionally, these mice display early motor impairment, probably indicating underlying neuropathological alterations.

Expression of Ifn- γ does not result in neuroinflammation in mice deficient in *Ifngr1*

From a mechanistic perspective, we wanted to investigate whether the unique neurodegenerative phenotype in the Ifn- γ mouse model is mediated via canonical Ifn- γ signaling in the brain. Ifn- γ signals by binding the Ifn- γ receptor (Ifngr) which is composed of two ubiquitously expressed subunits Ifngr1 and Ifngr2, both of which are essential for Ifn- γ signaling. Upon Ifn- γ binding, Ifn- γ receptors oligomerize and activate the Janus kinases (Jak1 and Jak2) by trans-phosphorylation leading to the phosphorylation of the signal transducer and activator of transcription 1 (Stat1), the intracellular signaling mediator. Phospho-Stat1 then dimerizes and translocates to the nucleus where it regulates gene expression by binding to promoter-associated γ -activated sequence elements in Ifn- γ regulated genes [22]. Both *Ifngr1*^{-/-} [10] and *Stat1*^{-/-} [11] mice have been shown to be nonresponsive to Ifn- γ signaling, indicating that these intermediates are key components of the canonical Ifn- γ signaling pathway. We injected *Ifngr1*^{-/-} or *Stat1*^{-/-} neonatal mice with AAV-Ifn- γ or AAV-EGFP on neonatal day P2 and analyzed the mice for neuropathological alterations at 5 months of age. GFAP (Fig. 3) and Iba-1 (Fig. 4) immunohistochemistry shows that compared to the widespread astrogliosis observed in WT mice expressing Ifn- γ , *Ifngr1*^{-/-} injected with Ifn- γ does not show any inflammatory phenotype in all areas of the brain examined. This is consistent with the fact that absence of Ifngr1 renders mice unresponsive to Ifn- γ mediated immune challenges [10]. On the other hand, Ifn- γ expression in the CNS of *Stat1*^{-/-} mice leads to induction of astrogliosis (Fig. 3) and microgliosis (Fig. 4) when compared to WT control mice, though the extent of immune activation is overall lower compared to WT mice expressing Ifn- γ . Interestingly, compared to *Ifngr1*^{-/-} mice expressing Ifn- γ , the extent of astrogliosis in Ifn- γ expressing *Stat1*^{-/-} mice was noticeably more (Fig. 3B, $p < 0.01$, 1-way ANOVA) than the effect on microgliosis (Fig. 4B, $p = \text{ns}$, 1-way ANOVA). We also quantified the levels of GFAP proteins in Ifn- γ expressing *Ifngr1*^{-/-} or *Stat1*^{-/-} mice, which shows that Ifn- γ expression can lead to increased astrogliosis in the absence of Stat1 but not in the absence of Ifngr1 (Supplementary Figure 4). It has been shown previously that Ifn- γ -dependent signaling can occur in the absence of Stat1, resulting in induction of Ifn- γ -inducible genes, provided Ifngr1 and Jak1 are present [22, 23]. Thus, we surmise that the astrogliosis in AAV-Ifn- γ injected *Stat1*^{-/-} mice is due to Stat1-independent Ifn- γ signaling.

Ifn- γ expression in *Ifngr1*^{-/-} or *Stat1*^{-/-} does not lead to midbrain calcinosis or nigrostriatal degeneration

Next, we wanted to examine whether absence of either Ifngr1 or Stat1 would abrogate the basal ganglia calcinosis and nigrostriatal degeneration phenotypes induced by the CNS expression of Ifn- γ . We found that compared to WT mice injected with Ifn- γ that display nigrostriatal degeneration shown by reductions in DARPP32 and Th immunostaining at 5 months of age [8] (Supplementary Figure 3A–C), neither *Ifngr1*^{-/-} or *Stat1*^{-/-} mice expressing Ifn- γ showed any loss of DARPP32 (Fig. 5A, B, C) or Th levels by immunohistochemistry (Fig. 6A–C) or immunoblotting (Supplementary Figure 4). No basal ganglia calcinosis was detected in *Ifngr1*^{-/-} or *Stat1*^{-/-} mice expressing Ifn- γ at 5 months of age, in contrast to WT mice (Fig. 5D). Overall, these results demonstrate that the unique neurodegenerative phenotype induced by Ifn- γ expression in the nigrostriatal pathway of WT mice requires the presence of Ifngr1 and Stat1.

Discussion

Here, using the AAV-Ifn- γ mouse model, we provide the first evidence of a direct causal link between canonical Ifn- γ signaling and selective neurodegeneration in the nigrostriatal pathway, a brain area that is selectively affected in Parkinsonian disorders [24]. We find that expression of Ifn- γ in WT mice is associated with early induction of neuroinflammation and motor deficits. Consistent with our original report [8], we provide evidence that this is associated with global induction of Stat1 phosphorylation, basal ganglia calcinosis and age-progressive nigrostriatal degeneration. Using mouse models that are deficient in key canonical intermediates of the Ifn- γ signaling pathway, we further show that *Ifngr1*^{-/-} mice and *Stat1*^{-/-} mice are completely resistant to Ifn- γ induced basal ganglia calcinosis and nigrostriatal degeneration, indicating that Ifngr1 and Stat1 are required for this unique phenotype. When expressing Ifn- γ in the absence of Stat1, we observed elevated astrocytosis but did not observe any calcinosis or nigrostriatal degeneration. This, together with our previous data that IL-6 induced neuroinflammation does not lead to midbrain pathologies [8], strongly suggests that canonical Ifn- γ signaling via Stat1 is specifically required to drive selective nigro-striatal degeneration. Future studies are needed to address whether this selective neurodegenerative phenotype are sensitive to non-cell autonomous events or result from a direct impact on neuronal homeostasis as a result of Ifn- γ induced signaling cascade.

How Ifn- γ signaling can result in such stark and selective neurodegenerative phenotype remains to be resolved. Although numerous hypotheses have been proposed to account for differential neuronal vulnerability to various pathological factors, such as neuroinflammation, there remains a poor understanding of why one population of cells is more vulnerable to a given neurodegenerative process than another. One of the most intensively studied neuronal populations with respect to differential neuronal vulnerability are the DA neurons within the SN [2]. Selective loss of DA input to the striatum and dysfunction of DA neurons in the SN result in a severe movement disorder with Parkinsonian characteristics [24]. Though neuroinflammation has been proposed to contribute to Parkinson's disease pathogenesis in humans and preclinical models, the exact etiology is unclear [2]. Interestingly, acute viral infections that can increase the inflammatory cytokine levels in the periphery can lead to a Parkinsonian syndrome, as observed in survivors of the Spanish flu of 1918 who were afflicted with 'encephalitis lethargica' [25] and indeed, this could be recapitulated in rodents infected with the virulent H5N1 influenza virus [26]. In preclinical models, nigral cells have been shown to be susceptible to LPS, a potent inducer of neuroinflammation [27]. Given that LPS has multiple targets, such data reinforce the generalized notion that DA neurons are sensitive to neuroinflammation but exactly which immune signaling pathway triggers selective neuronal death remains enigmatic. Our data indicates that simple neuroinflammation is not sufficient to cause SN degeneration as Ifn- γ induced Stat1-independent neuroinflammation does not lead to DA degeneration. Ifn- γ can also signal through non-canonical intermediates (not requiring Stat1) [28]. Moreover, expression of IL-6, an inflammatory cytokine unrelated to Ifn- γ , using a similar AAV paradigm also does not cause nigrostriatal degeneration [8]. Thus, our previous data [8], coupled with the present study, provide definitive and

compelling in vivo evidence that canonical Ifn- γ signaling through Ifngr1/Stat1-mediated canonical signaling mediates this robust, selective and progressive degenerative process. Consistent with our observations, *Ifn- γ ^{-/-}* mice are less susceptible to MPTP-induced DA degeneration [29]. Additionally, DA neurons in culture are resistant to rotenone-induced death in the presence of microglia lacking Ifngr1 [29]. Together these data suggests that blocking Ifn- γ signaling can have a neuroprotective effect on DA neurons. Interestingly, a dinucleotide CA repeat polymorphism in the first intron of the IFN- γ gene that leads to increased production of IFN- γ is associated with late onset Parkinson's disease [30]. Still, a major unanswered question remains: why are the neurons in the SN and basal ganglia so uniquely vulnerable to only Ifn- γ ? Cell culture studies demonstrated that selective neurotoxicity can be mediated via Ifn- γ induced phosphorylation of GluR1 in the presence of Jak2/Stat1 and PKA [31]. Additionally, Ifn- γ induced neuronal MHC-I expression can also selectively sensitize DA neurons to T cell mediated cytotoxicity [32]. Since Ifn- γ receptors are expressed relatively ubiquitously [33], we posit that regional variations in the density, distribution or activation capacity of Ifn- γ inducible genes may differentially regulate the stressor threshold to Ifn- γ in various brain regions. It is also possible that differential response of individual cell types (immune and non-immune) to Ifn- γ determines this threshold [5]. Together, these factors may be a critical determinant of selective vulnerability of DA neurons to Ifn- γ signaling.

Interestingly, Ifn- γ induced selective neurodegeneration closely parallels the spatial distribution of calcified deposits, although the mechanistic link remains to be studied. It is well-known that DA neurons are at one extreme of the phenotypic range of Ca²⁺ buffering, exhibiting the highest basal levels of mitochondrial oxidant stress and free cytosolic Ca²⁺ [24]. These features might increase their sensitivity to non-cell autonomous inflammatory stressors in their environment that can exacerbate excitotoxic stress leading to disturbed Ca²⁺ buffering and cellular homeostasis.

Conclusion

Overall, our data shows that DA neurons are uniquely susceptible to canonical Ifn- γ signaling, recapitulating the selective neurodegenerative features reminiscent of Parkinsonian syndromes. Future studies will require a systems-based evaluation of Ifn- γ signaling pathways that are differentially manifested in DA neurons and other neuronal or non-neuronal subtypes to fully understand the mechanisms underlying cytokine-mediated selective neuronal vulnerability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Expression of Ifn- γ in wild type mice results in neuroinflammation, motor deficits, basal ganglia calcinosis and dopaminergic neurodegeneration.
- This mouse phenocopies the Parkinsonian disorder, Fahr's disease.
- Deletion of Ifngr1, the primary receptor for Ifn- γ , results in complete abrogation of neuroinflammation and nigrostriatal degeneration in response to Ifn- γ expression
- Deletion of Stat1, component of the type II IFN receptor-signaling complex, results in neuroinflammation but no nigrostriatal degeneration in response to Ifn- γ expression
- Canonical Ifn- γ signaling via Ifngr1 and Stat1 is required for Parkinsonian neurodegenerative pathology

Figure 1. CNS expression of Ifn- γ leads to early inflammatory activation
WT mice were injected with AAV-Ifn- γ or rAAV-EGFP (Control) on neonatal day P2 and aged.

A. Heat map depicting hierarchical clustering of samples following focused transcriptomic profiling using NanoString arrays. The ages and treatment conditions of mice used are mentioned in the bottom of the heat map. n=2–3/group

B. Principal component analysis of gene expression changes in Ifn- γ expressing mice and 5 month old control mice. K-Means analysis of gene expression changes shows that Ifn- γ expressing mice cluster separately from control mice. n=2–3/group

C. Volcano plot and associated table show DEG in 5 month old Ifn- γ expressing mice compared to age-matched control mice. n=2–3/group. Multiple t test with Q=0.01. Orange dots represent log(2) fold change of >2.

D. Analysis of upregulated genes (fold change>5) were classified into KEGG pathways showing that multiple pathways related to altered immunity are significantly perturbed in AAV-Ifn- γ injected mice. n=2–3/group.

Figure 2. Ifn- γ expressing mice show early motor deficits

AAV-Ifn- γ mice were significantly affected in their rotarod performance compared to AAV-EGFP (control) expressing mice at all ages examined. AAV-Ifn- γ mice were motor impaired as early as 1 month of age. Mice were trained on Day 1 and subsequently for the next 3 days (Days 2, 3 and 4); mice were tested with 4 trials per day. These trials were averaged and presented as mean distance travelled ($\pm$ s.e.m). $n=6-10$ /group; *** $p<0.001$, ** $p<0.01$, * $p<0.05$; 1-way Anova with Tukey's Test.

Figure 3. Ifn- γ induced astrocytosis is abrogated in mice constitutively lacking *Ifngr1* but not in *Stat1* null mice

WT, *Ifngr1*^{-/-} or *Stat1*^{-/-} mice were injected with rAAV-Ifn- γ or rAAV-EGFP (Control) on neonatal day P2 and aged till 5 months. Representative GFAP immunohistochemistry from different brain regions show that compared to WT mice expressing Ifn- γ , *Ifngr1*^{-/-} mice do not show any detectable astrocytosis while *Stat1*^{-/-} mice show increased levels of GFAP immunostaining (A). The immunostaining was quantified from three sections from each mouse brain using Aperio imaging algorithms (B). AAV-Ifn- γ injected *Ifngr1*^{-/-} or *Stat1*^{-/-} mice do not show basal ganglia calcinosis. Arrows indicate midbrain calcinosis in WT mice expressing Ifn- γ . Insets display higher magnification of the corresponding panel. n=5 mice/group. Scale Bar, 500 μ m; 100 μ m (inset). ****p<0.001, **p<0.01, *p<0.05; 1-way Anova with Tukey's Test.

Figure 4. Ifn- γ induced microgliosis is completely abrogated in mice constitutively lacking *Ifngr1* and partially in *Stat1* null mice

WT, *Ifngr1*^{-/-} or *Stat1*^{-/-} mice were injected with rAAV-Ifn- γ or rAAV-EGFP (Control) on neonatal day P2 and aged till 5 months. Representative Iba-1 immunohistochemistry from different brain regions show that compared to WT mice expressing Ifn- γ , *Ifngr1*^{-/-} mice do not show any detectable neuroinflammation whereas *Stat1*^{-/-} mice have slightly increased levels of microgliosis (A). The immunostaining was quantified from three sections from each mouse brain using imaging algorithms (B). Insets display higher magnification of the corresponding panel. n=5 mice/group. Scale Bar, 500 μ m; 100 μ m (inset). ****p<0.001, **p<0.01, *p<0.05; 1-way Anova with Tukey's Test.

Figure 5. Expression of Ifn- γ does not lead to loss of DARPP32 levels or midbrain calcinosis in *Ifngr1*^{-/-} or *Stat1*^{-/-} mice

A–C. Representative DARPP32 immunostaining from whole brain (A) and magnified image from SN (B) shows that expression of Ifn- γ in *Ifngr1*^{-/-} or *Stat1*^{-/-} mice does not lead to loss of DARPP32 immunoreactivity in contrast to Ifn- γ expressing WT mice that exhibit nigro-striatal degeneration. The levels of DARPP32 immunoreactivity has been quantified by Aperio imaging algorithms (C). n=6 mice/group. **p<0.01, ***p<0.001, 1-way Anova. Scale Bar, 500 μ m (A), 25 μ m (B).

D. Magnified image from DARPP32 stained brains show that while WT mice expressing Ifn- γ develop robust calcinosis in the thalamus, neither *Ifngr1*^{-/-} or *Stat1*^{-/-} mice injected with AAV-Ifn- γ show this unique phenotype. Scale Bar, 25 μ m.

Figure 6. Expression of Ifn- γ does not lead to loss of TH levels in *Ifngr1*^{-/-} or *Stat1*^{-/-} mice
A–C. Representative TH immunostaining shows no loss of nigro-striatal pathway in *Ifngr1*^{-/-} or *Stat1*^{-/-} mice injected with AAV-Ifn- γ compared to WT mice injected with AAV-Ifn- γ (A). Magnified images from the SN show that nigral DA neurons are intact in *Ifngr1*^{-/-} or *Stat1*^{-/-} mice injected with AAV-Ifn- γ compared to WT mice injected with AAV-Ifn- γ (B). The levels of TH immunoreactivity has been quantified by imaging algorithms (C). n=6 mice/group. ****p<0.001, 1-way Anova. Scale Bar, 500 μ m (A), 25 μ m (B).