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SVILUPPO E SPERIMENTAZIONE DEI FARMACI ANTIVIRALI

Ciclo XXIII

Type I Interferon-induced neuronal damage: A study of the cellular and molecular mechanisms mediating interferon neurotoxicity

Settore scientifico-disciplinare: 05-Scienze Biologiche

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Abbreviations:

ATF, activating transcription factor;
BDNF, brain-derived neurotrophic factor;
BSA, bovine serum albumin;
bZIP, basic-leucine zipper;
C/EBP, CCAAT/enhancer binding protein;
CHOP, C/EBP homologous protein;
CREB, cyclic AMP responsive element binding protein;
CRH, corticotrophin-releasing hormone;
DAPI, 4',6-diamidino-2phenylindole dihydrochloride;
eIF2α, eukaryotic initiation factor 2α;
ERK, extracellular signal-regulated protein kinase;
FADD, fas-activated death domain;
FCS, fetal calf serum;
FITC, fluorescein isothiocyanate;
GAS, gamma activation sequence;
GSK, glycogen synthase kinase;
HIV, human immunodeficiency virus;
HPA, hypothalamus-pituitary-adrenal;
HSF-1, heat shock factor 1;
IDO, indoleamine 2,3-dioxygenase;
IFN, interferon;
IGF-I, insulin-like growth factor-1;
IKK, inhibitor of NFκB kinase;
IL, interleukin;
IPS1, IFNβ-promoter stimulator 1;
IRF9, IFN response factor 9;
IRS-1, insulin receptor substrate-1;
ISG, interferon-stimulated gene;
ISGF3, interferon-stimulated gene factor 3;
ISRE, interferon stimulated regulatory element;
JAK, Janus kinase;
JNC, Jun N-terminal kinase;
KSHV, Kaposi's sarcoma-associated virus;
LPS, lipopolysaccaride;
MAPK, mitogen-activated protein kinase;
MDA5, melanoma differentiation associated protein 5;
MEF2D, myocyte enhancer factor 2D;
MKP, MAPK phosphatase;
MLK3, mixed lineage kinase 3;
MS, multiple sclerosis;
Mx, mixovirus;
NF160/200, neurofilament 160/200;
NFκB, nuclear factor-κB;
NSE, neuron specific enolase;
2'-5'-OAS, 2′-5′-oligoadenylate synthetase;
2'-5'OA, 2'-5' oligoadenylate;

P6, pyridone 6;
PAGE, polyacrylamide gel electrophoresis;
PARP, poly-(ADP ribose) polymerase;
PBS, phosphate-buffered saline;
PEG-IFN, pegylated-IFN;
PKR, double-stranded RNA-activated protein kinase;
PI, propidium iodide;
PIAS, protein inhibitors of activated STAT;
PI3K, phosphatidylinositol 3-kinase;
PMA, phorbol 12- myristate 13-acetate;
PMSF, phenylmethylsulphonyl fluoride;
PTP, protein tyrosine phosphatase;
RA, all-trans-retinoic acid;
RIG-I, retinoic acid inducible gene I ;
ROD, relative optical density;
SDS, sodium dodecyl sulphate;
siRNA, small interfering RNA;
SOCS, suppressor of cytokine signaling;
STAT, signal transducer and activator of transcription;
SUMO, small ubiquitin-related modifier;
TH, tyrosine hydroxylase;
TNF-α, tumor necrosis factor α;
TRIF, TLR adapter molecule;
TrK, tropomyosin receptor kinase;
TRL, toll-like receptor;
TUNEL, terminal transferase dUTP nick end-labelling;
Tyk2, tyrosine kinase 2;
VDAC, voltage-dependent anion channel;
VV, vaccinia virus.

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ABSTRACT

Type I interferons (IFNs) are known to cause neuropsychiatric side effects, including cognitive and mood disturbances, through mechanisms still not completely defined. To gain more information about type I IFN neurotoxicity, I investigated whether these cytokines could act directly on neuronal cells and regulate intracellular signaling pathways involved in cell death. In primary cultures of mouse cortical neurons acute exposure to IFN-β induces a marked tyrosine phosphorylation of signal transducer and activator of transcription (STAT) 1 and 3, whereas long-term exposure to the cytokine increased the number of neuronal cells displaying an increased labeling for cleaved caspase 3. In human SH-SY5Y neuroblastoma cells, used as a neuronal cell model, type I IFNs rapidly stimulated the tyrosine phosphorylation of Janus kinase (JAK) and STAT1, STAT3 and STAT5 and this response was antagonized by blockade of type I IFN receptor. Prolonged exposure to IFN-β induced apoptotic cell death accompanied by cytochrome C release, cleavage of caspase-9, -7, -3 and poly-(ADP ribose) polymerase (PARP), DNA fragmentation and p38 mitogen-activated protein kinase (p38 MAPK) phosphorylation. JAK inhibition reduced IFN-β-stimulated Tyk2 and STAT1 phosphorylation, STAT1 transcriptional activity, induction of double-stranded RNA-activated protein kinase (PKR), caspase cleavage and p38 MAPK phosphorylation. PKR induction was associated with enhanced PKR activity and chemical inhibition of PKR reduced IFN-stimulated caspase activation. Moreover, long-term IFN-β treatment led to down-regulation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling and IFN-β-induced apoptosis was attenuated in cells expressing constitutively active Akt. The proapoptotic effect of IFNs was markedly attenuated by pharmacological blockade of either glycogen

synthase kinase-3 (GSK-3) or p38 MAPK. In retinoic acid-differentiated SH-SY5Y cells, treatment with IFN-β inhibited BDNF-induced regulation of Akt and GSK-3β, activation of extracellular signal-regulated protein kinase 1 and 2 (ERK1/2), phosphorylation of tyrosine hydroxylase, and neuritogenesis. Thus, type I IFNs can directly impair neuronal survival by regulating multiple mechanisms, including activation of the canonical JAK-STAT signaling and the ancillary p38 MAPK pathway and down-regulation of the PI3K/Akt and ERK1/2 signaling systems. The final outcome of these molecular events is the activation of the apoptotic program and impaired neuronal differentiation in response to neurotrophic stimuli. It is proposed that these actions directed on neuronal cells may contribute to the neuropsychiatric disturbances elicited by these cytokines.

BACKGROUND

Interferons (IFNs) are a family of structurally related cytokines with a hallmark function of antiviral activity. The characterization of the interferon genes, their expression, and their function was advanced with the development of novel techniques in molecular and cellular biology. The biomedical interest in IFN-α and IFN-β is linked to their therapeutic efficacy against viral infections, different types of cancer and neuroinflammatory diseases, particularly multiple sclerosis. However, these cytokines induce a number of peripheral and central adverse effects, which compromise tolerability and often limit the use of high dosage required to control cancer or viral disease. Among the most serious central side effects there are anxiety, confusion, mania, psychosis, cognitive deficits, depression and suicidal behaviour, through mechanisms still not completely defined. Depression has been reported to occur in 7–35% of patients depending on the duration of IFN treatment. Although the neuropsychiatric effects most often subside after IFN dosage reduction or discontinuation, there are reports that IFN neurotoxicity may persist for months or years after IFN withdrawal. The mechanisms that mediate the IFN neurotoxic effects remain unclear. Several physiological mechanisms have been proposed for the neurotoxic effects of IFNs, including actions mediated through neuroendocrine, neurotransmitter and cytokine pathways.

It has been suggested that glia-derived factors may play a role in the alterations of monoamine neurotransmission and induction of neuropsychiatric side effects. However, an additional possibility is that type I IFNs may exert toxic effects directly on neuronal cells. Gene chip analysis of RNA from primary brain cell culture treated with IFN-α indicated that neurons are very responsive target cells for IFNs. At the

present, little is known on the expression of functional type I IFN receptors in human neuronal cells and on the ability of these cytokines to affect neuronal cell viability by regulating signaling pathways governing cell survival and apoptosis.

1. INTRODUCTION

1.1 The interferon proteins

The discovery of interferons (IFNs) dates almost 50 years ago when Alick Isaacs and Jean Lindenmann described for the first time the phenomenon of the viral interference (Isaac and Lindenmann, 1957). Their discovery stemmed from experiments in which the exposition of chicken embryo cells to a strain of heat-inactivated influenza virus A stimulated cells to produce a secreted factor, which, when added to fresh cells, interfered with the replication of an active preparation of that same virus. This experiment led to the discovery of the active component of the most common type of viral interference which was termed interferon. Released IFNs were shown to act on specific receptors expressed on the cell membranes to induce the synthesis of proteins which block virus reproduction and diffusion in the organism. These observations led to test IFNs as potential therapy for infections by RNA and DNA viruses. Moreover, different studies demonstrated that IFNs not only regulate resistance to viral infections but also exert antiproliferative and immunomodulatory effects, suggesting their validity in cancer and autoimmune diseases (for a recent review, see Borden *et al*., 2007).

In humans, seven IFNs species (termed α, β, Є, κ, ω, ν, and γ) are found (Pestka *et al*., 2004) (Table 1) and are grouped into three classes called type I, II and III IFNs. The IFNs-α, -β, -Є, -κ, -ω, -ν are classified as Type I, whereas IFN-γ is the only Type II IFN. Type III IFNs comprises IFN-like proteins, such as interleukin (IL)-28A, IL-28B and IL-29 and can be induced in many cells. Unlike IFN-α and -β, the type III IFN receptor shows a limited tissue distribution (Meager *et al*., 2005; Mennechet & Uze, 2006; Zhou *et al*., 2007). These three classes are defined by their differences in

amino acid sequences, physicochemical properties, induction by different agents (such as viruses, bacteria, bacterial products, polymers, low molecular weight compounds and antigens or mitogens) and cell source.

Types	Subtypes of IFNs	Receptors	Gene locus[a]	Amino acid residues	Molecular weight (kDa)	Expression pattern
I	IFN-α	IFNAR-1/IFNAR2	9p21	165–166[b]	15–23	Ubiquitously expressed
	IFN-β	IFNAR-1/IFNAR2	9p21	166[b]	15–23	Ubiquitously expressed
	IFN-δ[c]	IFNAR-1/IFNAR2	None	170	20	Trophoblasts
	IFN-ε	IFNAR-1/IFNAR2	9p21	208	24.4	Uterus, ovary
	IFN-ω	IFNAR-1/IFNAR2	9p21	172[b]	20–23	Leukocytes
	IFN-κ	IFNAR-1/IFNAR2	9p21	180[b]	24.5	Selectively expressed in epidermal keratinocytes
	IFN-τ[d]	IFNAR-1/IFNAR2	None	191[b]	20–22	Trophoblasts
	IFN-ζ[e]	IFNAR-1/IFNAR2	None	161[b]	20	Spleen, thymus, lymph node
II	IFN-γ[f]	IFNGR-1/IFNGR2	12q24.1	146[b]	34	Activated T cell, macrophage, NK cell
III	IFN-λ1 (IL-29)	IL-28Rα/IL-10R2	19q1	200	20–33[g]	Ubiquitously expressed
	IFN-λ2 (IL-28A)	IL-28Rα/IL-10R2	19q1	200	22	Ubiquitously expressed
	IFN-λ3 (IL-28B)	IL-28Rα/IL-10R2	19q1	196	22	Ubiquitously expressed

a. Human.
b. Signal peptides are not included.
c. Found in pigs and cattle only.
d. Found in ruminants only.
e. Found in mice only.
f. Acts as a homodimers.
g. Due to its glycosylation.
Data about molecular properties were obtained from the following NCBI web site: http://www.ncbi.nlm.nih.gov except for IFN-λs (Kotenko *et al.*, 2003).

Table 1

Human IFN and IFN-like proteins
from: Takaoka and Yanai, 2006

Each IFN, with the exception of IFN-α, is a product of a single gene and thus present as a single protein or, in some cases, as few proteins differing by only few amino acid residues and considered allelic variants.

So far, most of the studies focused on IFN-α, IFN-β and IFN-γ. In humans, there are at least 12 different subtypes of IFN-α (besides one or two allelic variants for each subtype) that are encoded by 13 distinct genes (Table 2). These subtypes display high sequence homology (80%), whereas the identity with the other type I IFN is less, ranging from 60 to 30%.

Type I IFNs, including all IFN-α subtypes and IFN-β, are encoded by intron-less genes and are characterized by a secondary structure, comprising 5 α helices and 2 disulfide bridges between Cys1/99 and 29/139, and a tridimensional conformation where the α helices are collected in a bundle. Type I IFNs share similar biological activities as well as biophysical properties, such as retention of biological activity at low pH, which is not showed by IFN-γ. Accordingly, the sequence homology with IFN-γ is only 10%.

The existence of a number of different type I IFNs acting on the same receptor raises the question of why this multigenic family has been conserved during evolution. Although the answer has not yet been reached, IFNs show important quantitative differences in their biological activities, suggesting distinct roles in innate immunity. A possible explanation for this diversity is that the mode of interaction of each IFN with the receptor might be different, possibly triggering intracellular signals that differ quantitatively and qualitatively among cell types and even in the same cell.

Gene	Protein
IFNA1	IFN-αD, IFN-α1
IFNA2	IFN-α2a, IFN-α2b, IFN-α2c
IFNA4	IFN-α4a, IFN-α4b
IFNA5	IFN-αG, IFN-α5, IFN-α61
IFNA6	IFN-αK, IFN-α6, IFN-α54
IFNA7	IFN-αJ, IFN-αJ1, IFN-α7
IFNA8	IFN-αB2, IFN-αB, IFN-α8
IFNA10	IFN-αC, ΨIFN-α10, ΨIFN-αL, IFN-α6L
IFNA13	IFN-α13 (sequence identical to IFN-α1)
IFNA14	IFN-αH, IFN-αH1, IFN-α14
IFNA16	IFN-αWA, IFN-α16, IFN-αO
IFNA17	IFN-αI, IFN-α17, IFN-α88
IFNA21	IFN-αF, IFN-α21
IFNAP22*	YIFN-aE

Table 2

Human IFNα genes (13) and proteins (12)
* pseudogene

1.2 The IFNAR receptor

The conservation of the multitude of type I IFNs, throughout evolution of the mammals is consistent with the unique functional roles for each of the type I IFN (Borden *et al*., 2007). IFN-α and IFN-β exert their biological actions by binding to and activating a common cell-surface heterodimeric receptor, named IFN-α receptor (IFNAR), that consists of IFNAR1 and IFNAR2 chains.

IFNAR1 is a glycoprotein consisting of an extracellular portion, containing four globular domains with two-layer β-structural conformation, a 21 amino acid transmembrane segment and a 100 amino acid cytoplasmic domain (Origani *et al*., 2001).

The IFNAR2 chain shows three different forms, the short form (IFNAR-2a), the soluble form (IFNAR-2b) and the long form (IFNAR-2c). The long form is composed of 515 amino acids and works as a functional receptor for IFN-α and IFN-β.

Early studies on type I IFN binding prior to the cloning of the receptor chains have indicated that most cell types display large variation in the number of binding sites (200–10,000/cell) and binding affinities. In particular, Scatchard analyses of binding isotherms identified two binding sites, one with a low (μM) and the other with a high affinity (nM–pM). This type of binding suggested the existence of a multicomponent receptor structure formed by a high affinity binding chain (termed the α chain) and a β or signal-transducing chain, that had a low binding affinity. The β chain, however, increased the affinity of IFNs for the α chain from moderate (nM) to high affinity (pM). More recent investigations using recombinant IFNAR extracellular domains tethered to lipid membranes have shown that various type I IFNs bind to IFNAR2 with affinities

predominantly in the nM range (from 0.1 to 1000 nM), whereas they bind to IFNAR1 with affinities mostly in the μM range (from 0.05 to 10 μM).

How various type I IFNs can display different biological activities by acting on a common receptor is a still unresolved issue.

It has been found that type I IFNs interact with the receptor differently and this property may explain, at least in part, their different biological activities. A number of studies have investigated the residues of IFNAR2 involved in ligand interactions with IFN-α2 and IFN-β. The ligand binding site of human IFNAR2 is composed largely of aliphatic hydrophobic amino acids. Three highly conserved residues Thr^{44}, Met^{46}, and Lys^{48} form the core of the IFNAR2 ligand binding domain, whereas residues, His^{76}, Glu^{77}, Tyr^{81}, Trp^{100}, Ile^{103}, and Asp^{106} also facilitate IFN-α2 binding. These residues are predicted by NMR to form an extensive and largely aliphatic hydrophobic patch on the surface of IFNAR2. The interaction of IFN-β is predicted to be different from that of IFN-α, which involves IFNAR2 residues Ile^{45} and Trp^{100}, with minor contributions from Thr^{44}, Met^{46}, Ser^{47}, and Ile^{103} (Fig. 1). The sequence differences between type I IFNs result in distinct binding affinities with each IFNAR chain with consequent possible differences in biological activities. Thus, analysis of IFNs binding to IFNAR extracellular domains tethered on solid-supported membranes indicated a critical role of the absolute affinity values toward IFNAR1 for antiproliferative activity (Jaks *et al*., 2007). An IFN-α2 mutant optimized for IFNAR1 binding was also found to display enhanced antitumor activity (Kalie *et al*., 2007). Other studies using a panel of mutations targeting the IFN-α2 binding site to IFNAR2 have confirmed the key role of IFNAR1 for the cytostatic effects, and have proposed that the differential biological activity of type I IFNs may result from the stability of the

IFN-IFNAR ternary complex, rather than the affinity of a given IFN for the individual receptor chains (Kalie *et al*., 2008).

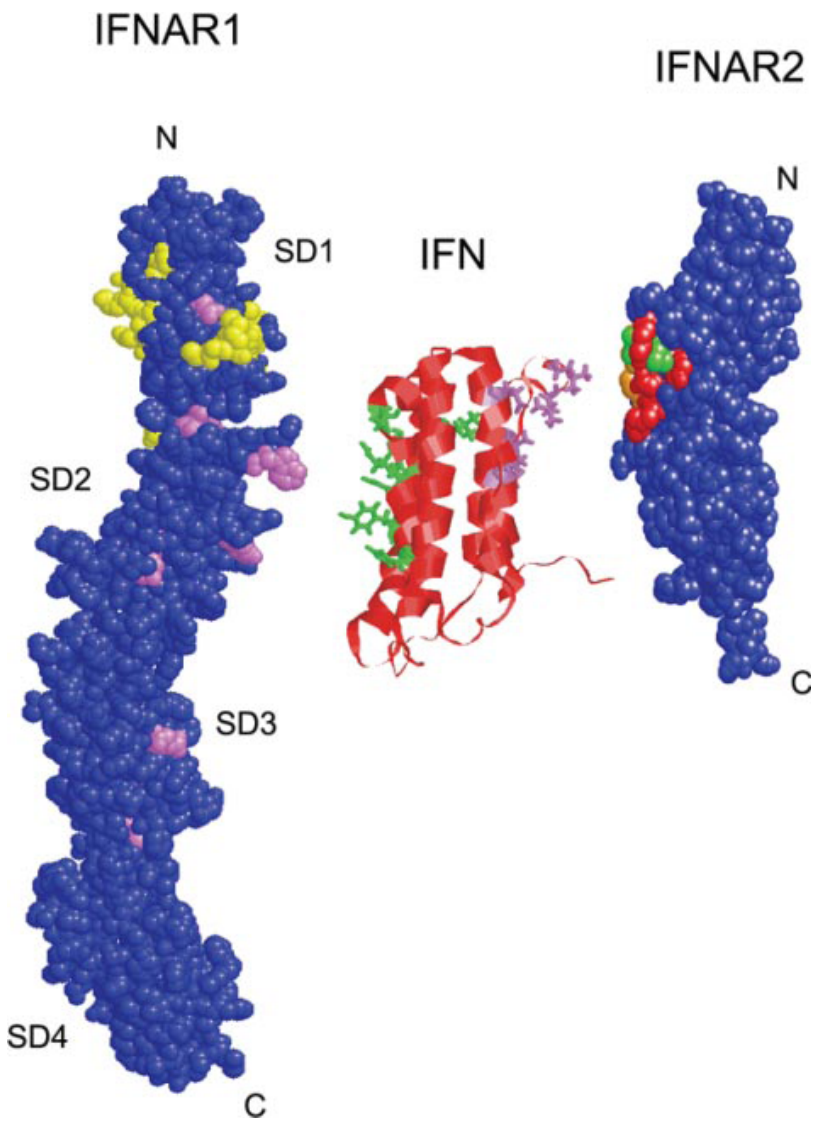


Fig. 1

The type I IFN receptor ternary complex. Amino acid residues of IFN-α2 involved in IFNAR1 interactions are shown in *green* and those involved in IFNAR2 interactions are shown in *violet.*
from: de Weerd et al., 2007

1.3 The JAK-STAT signaling pathway

The binding of type I IFNs to the heterodimeric receptor induces oligomerization. The cytoplasmic tail of IFNAR1 and IFNAR2 is pre-associated with tyrosine kinase 2 (Tyk2) and JAK1, respectively, which belong to the Janus family of tyrosine kinases (JAK). Under basal conditions, the transcription factor STAT (Signal Transducers and Activators of Transcription) 2 is bound to the IFNAR2 chain and is weakly associated with STAT1 (Stancato *et al.*, 1996; Precious *et al.*, 2005a). IFN binding to the receptor induces the dimerization and a conformational change, such that Tyk2 phosphorylates tyrosine 466 on IFNAR1, creating a strong docking site for STAT2. Tyk2 then phosphorylates STAT2 on tyrosine 690, and STAT1 is phosphorylated by JAK1 on tyrosine 701; the phosphorylated STAT1 and STAT2 can thus form a stable heterodimer. Phosphorylation of STAT1, its subsequent dimerization with STAT2 and the simultaneous phosphorylation of STAT2 inactivate the dominant constitutive nuclear export of STAT2 (Frahm *et al.*, 2006), so that the dimers become transported into and retained in the nucleus until their dephosphorylation (reviewed by Reich and Liu, 2006). The STAT1–STAT2 complex associates with a p48 protein identified as IFN response factor 9 (IRF9), forming the interferon-stimulated gene factor 3 (ISGF3).

The complex ISGF3 translocates to the nucleus and initiates the transcription at IFN-stimulated response elements (ISRE), thus activating interferon-stimulated genes (ISG) (Fig. 2).

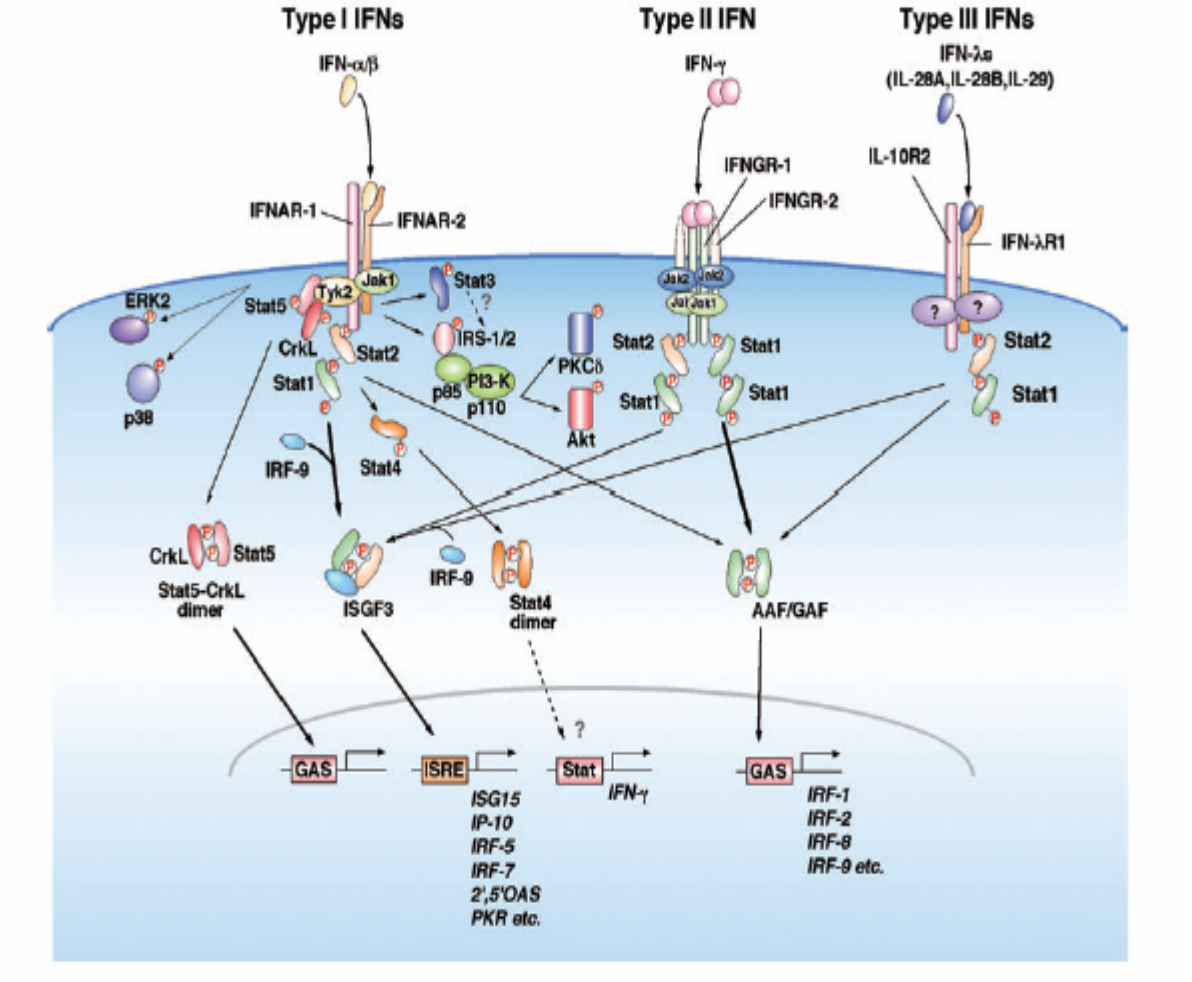


Fig. 2

Cellular signaling cascades triggered by IFNs
from: Takaoka and Yanai, 2006

The signaling pathways activated by type II and type III IFNs follow a very similar pattern to that developed in response to type I IFN (Zhou *et al.*, 2007). The IFN-γ receptor is also a heterodimeric glycoprotein, composed by two types of major subunits, IFNGR1 and IFNGR2, that are weakly pre-associated in unstimulated cells. The cytoplasmic domains of the IFNGR1 and IFNGR2 receptor subunits are associated with a different set of JAKs, JAK1 and JAK2, respectively. The binding of IFN-γ to the receptor subunits initiates the signaling cascade by triggering receptor dimerization, which brings JAK1 and JAK2 into close proximity. This results in the

activation of JAK2 which phosphorylates JAK1, thereby activating it. The activated JAKs phosphorylate a tyrosine-containing region in the C terminus of IFNGR1 (between tyrosine 440 and tyrosine 444), creating a pair of binding sites for STAT1. Two STAT1 molecules interact via their SH2 domains with IFNGR1 and are phosphorylated at tyrosine 701, resulting in their activation and dissociation from the receptor. The activated STAT1 molecules dimerize through SH2 domain–tyrosine phosphate recognition, forming a STAT1 homodimer. This homodimer translocates to the nucleus, binds to the gamma-activation sequence (GAS) and stimulates transcription of ISGs. Importantly, this sequence is distinct from the ISRE, and the binding of the STAT1 homodimer does not require IRF-9.

1.4 Negative regulation of JAK-STAT signaling

IFNs also regulate different regulatory molecules to inhibit JAK-STAT pathway, thus limiting the extent of signaling. The suppressor of cytokine signaling (SOCS) proteins, the PIAS (protein inhibitors of activated STATs) proteins and different tyrosine phosphatases can inhibit JAK-STAT signaling through distinct mechanisms (Fig. 3).

SOCS proteins are induced transcriptionally in response to cytokine stimulation, thereby providing a negative feedback circuit for IFN signaling modulation (Levy and Darnell, 2002; Rakesh and Agrawal, 2005). Although eight SOCS protein family members are known (SOCS1-7 and cytokine-inducible SH2 domain containing protein), the best characterized are SOCS1, SOCS2 and SOCS3. Each protein has a central SH2 domain, an amino-terminal domain of variable length, and a carboxy-

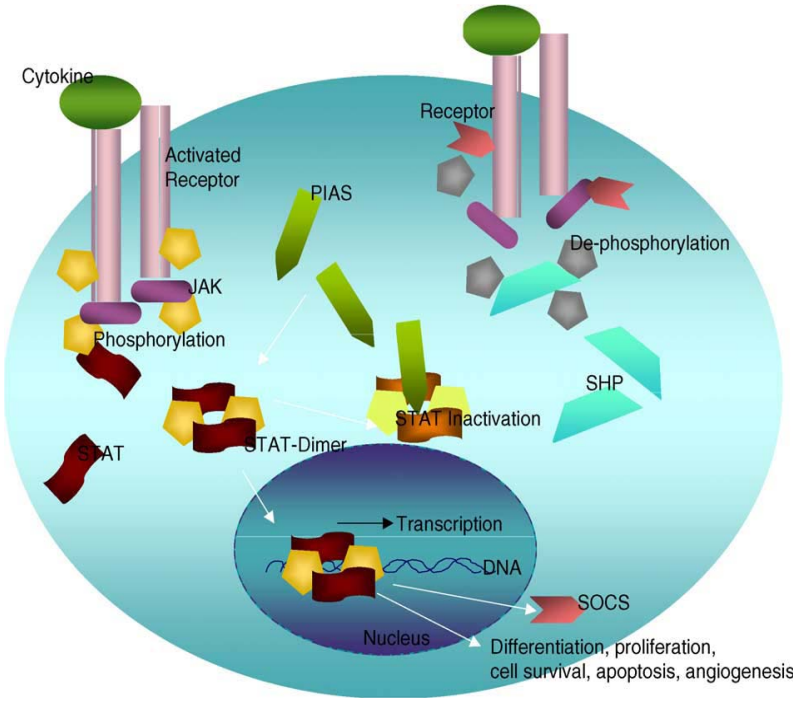


Fig. 3

Negative regulators of Jak/STAT signalling
from: Rakesh and Agrawal, 2005

terminal domain of 40-amino acid, known as the SOCS box. It has been proposed that SOCS proteins inhibit IFN signaling by binding to phosphorylated tyrosine residues on signaling intermediates, including receptor chains and JAKs, through their SH2 domain. SOCS family proteins, as well as other SOCS-box-containing molecules, probably function as E3 ubiquitin ligases and mediate the degradation of proteins associated through their amino-terminal regions. The SOCS box is also known to be important for stabilization and/or degradation of the SOCS1 and SOCS3 proteins and mice that lack only the SOCS box of SOCS1 exhibited inflammatory diseases similar to complete SOCS1-deficient mice (Zhang *et al*., 2001). The role of

the SOCS box in the function of each of the SOCS proteins has not been full elucidated.

PIAS are a group of constitutively expressed nuclear proteins that contain a zinc-binding cysteine-rich RING finger domain, an amino-terminal LXXLL co-regulator motif, a carboxy-terminal domain mediating TIF2 binding and a PINIT motif determining nuclear retention (Wong *et al*., 2004). Five members of this family have been identified: PIAS1, PIAS3 α and β, PIASx and PIASy. PIAS proteins possess E3-SUMO (small ubiquitin-related modifier) ligase activity promoting SUMO attachment to signaling proteins. Like protein ubiquitination, PIAS induced protein SUMOylation directs the substrates toward proteolytic degradation. PIAS proteins display some specificity for protein substrates, although not as high as that of ubiquitin ligases. PIAS1 and PIAS3 bind to STAT1 and STAT3, respectively, thereby preventing the interaction with DNA, whereas PIASx forms a complex with STAT4 (You *et al*., 1999). Besides inhibiting STATs, PIAS proteins can attack and repress other transcription factors, such as p53, c-Jun, androgen receptor, c-Myb and lymphoid enhancer factor 1 (LEF-1).

Several cytoplasmic tyrosine phosphatases, including SHP-1, SHP-2, CD45 and PTP1B, participate in the negative control of JAK-STAT pathway by dephosphorylating tyrosine residues of receptor chains, JAKs and other signaling intermediates. SHP-1 and SHP-2 bind with their SH2 domains to phosphotyrosine residues of different cytokine receptors.

Studies have demonstrated that the carboxy-terminus of IFNAR1 contains a conserved region of about 14 amino acids, which inhibits type I IFN signaling. This region also contains residues required for the recruitment of E3 ubiquitin ligases,

ubiquitination and degradation of the receptor (Kumar *et al*., 2004). Studies in knock-out mice with null mutation in IFNAR1 have demonstrated that this component is essential for α and β IFNs-induced survival against most viral infections, myelopoiesis, B and T cell-mediated immune responses and potent pro-inflammatory response.

1.5 Regulation of IFN expression

Type I IFNs are produced by different cell types, such as dendritic cells, macrophages, monocytes and fibroblasts, whereas IFN-γ is exclusively produced by T lymphocytes. IFNs are inducible proteins and their constitutive production is very low in unstimulated cells. Exposure to a number of potentially noxious factors, such as viruses, bacteria, or their products (i.e. DNA fragments, single- or double-stranded (ds) RNA, bacterial toxins and lipopolysaccarides (LPS)), triggers IFN synthesis. Recently, the identification of toll-like receptors (TLR) greatly advanced the understanding of the mechanisms of IFN induction. TLR are a family of transmembrane receptors present either on plasma membranes, with the recognition domain located extracellularly and the signal transduction domain facing the cytoplasm, or on endosomal membranes. In the latter case, the recognition domain is located inside the endosomes and the signal transduction domain oriented toward the cytoplasm in order to detect bacteria, viruses and their products following endosomal internalization (Fig. 4). In humans, at least 10 distinct TLR and 2 RNA helicases, retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated protein 5 (MDA5), play a role as soluble cytoplasmic virus receptors. At least 5 distinct TLR (4 of which are expressed in endosomes) are involved in IFN

induction following viral infection. The initial observation that dsRNAs were potent inducers of IFNs suggested that viruses were capable of inducing IFNs by producing dsRNAs.

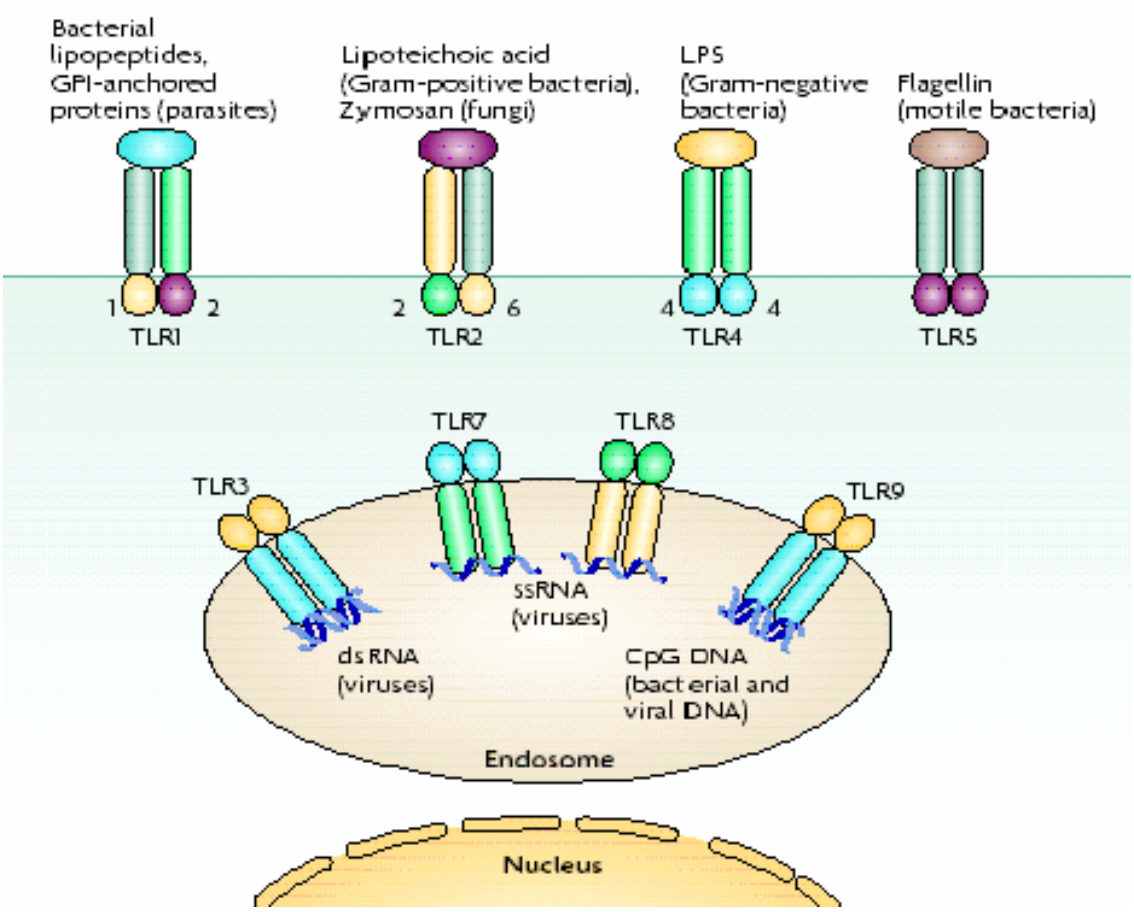


Fig. 4

Mammalian Toll-like receptors and their ligands
from: Borden et al., 2007

It is now evident that other virus gene products, besides dsRNA, can trigger IFN induction. Single-stranded (ss) RNAs can also be recognized, but only if they have 5'-triphosphates. While ds- and ss-RNAs are recognized by TLR3, RIG-I and MDA5, an additional receptor is present in cytoplasm for viral DNA (Yoneyama and Fujita, 2007). Other nucleic acids, such as ssRNAs, can act through TLR7 and TLR8,

whereas bacterial oligoDNAs act through TRL9. Bacteria LPS induces IFN by activating TLR4, whereas viral glycoproteins bind and activate different TLRs.

The molecular mechanisms mediating IFN induction have been particularly studied for TLR3 and RNA helicases (Fig. 5). The inducers are recognized by TRL3, which undergoes tyrosine phosphorylation, dimerization and complex formation with CD14, thereby triggering a signal cascade leading to the recruitment of the TLR adapter molecule (TRIF). For RIG-1 and MDA5A a mitochondrial protein, termed IFN-β-promoter stimulator 1 (IPS1), behaves as an adaptor. Both TRIF and IPS1 promote the recruitment of inhibitor of nuclear factor-κB (NFκB) kinase (IKK) and TANK-binding kinase (TBK1/IKKE). TBK1 then phosphorylates and activates IFN regulatory factor (IRF) 3 and IRF7, which act as transcription factors. Activated IRFs dimerize and traslocate to the nucleus (Fig. 5).

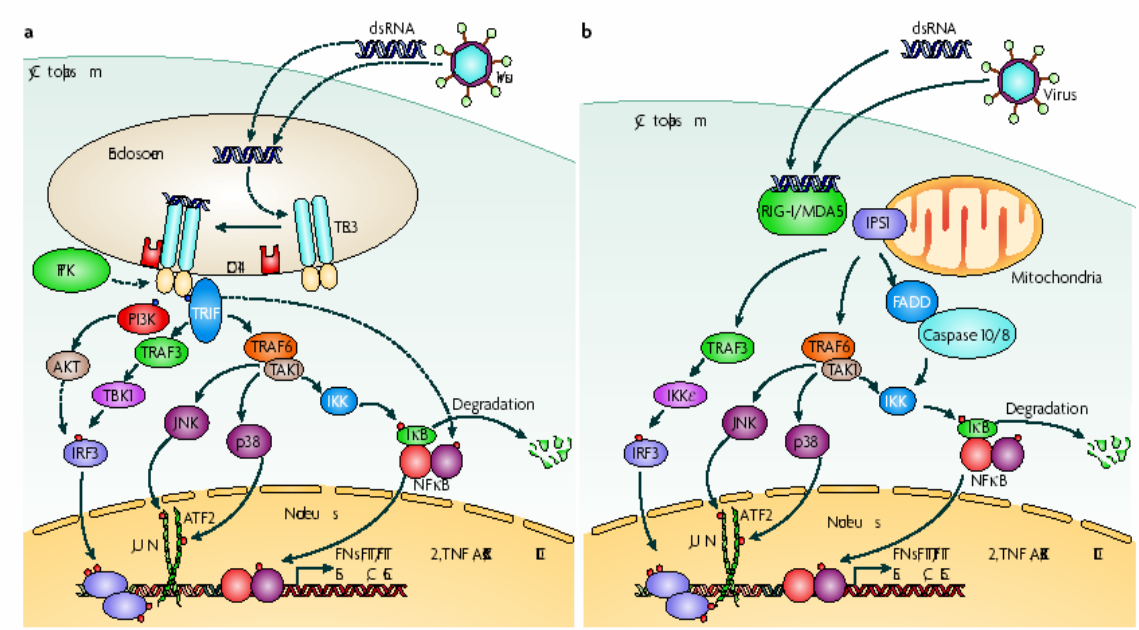


Fig. 5

Signaling mechanisms by TLR3 and RNA helicases RIG-1/MDA5
from: Borden et al., 2007

It has been shown that IRF3 full activation also requires phosphorylation by the protein kinase B/Akt, which is triggered by interaction of TLR3 with phosphatidylinositol 3-kinase (PI3K).

The IKK protein kinase complex phosphorylates IkB and releases it from NFκB, which then can acts as transcription factor. Interestingly, signals originated by the adaptor protein IPS1 also lead to activation of caspases 10/8 through fas-activated death domain (FADD), thus suggesting that viral RNA can cause both IFN induction and cell death.

In this complex molecular mechanisms of IFN induction, several key steps can be altered leading to the development of virus resistance to IFN action. For example, it has been shown that a hepatitis C virus-encoded protease can cleave IPS1 off the mitochondria membrane and therefore block RIG-1 and MDA5 activity (Foy *et al*., 2005). Proteins of hepatitis B virus, influenza virus, Ebola virus, papilloma virus and the human herpes Kaposi's sarcoma-associated virus (KSHV) can also block IFN induction or the action of IFNs by different mechanisms (Garcia-Sastre and Biron, 2006). For example, KSHV down-regulates one of the IFN-γ receptor chains, while the NS1 protein of influenza virus interacts with RIG-1.

The knowledge of the basic molecular mechanism allowed the development of small-molecule drugs that could induce IFN. CpG oligonucleotides, which are bacterial DNA oligodeoxynucleotide sequences including cytosine-guanosine, activate TLR9, whereas the quinolinamide imiquimod and its analogues stimulate TLR7 (Hemmi *et al*., 2000).

1.6 Mechanisms of IFN antiviral activity

IFN-stimulated genes (ISGs) comprise more than 300 genes that mediate the biological and therapeutic effects of IFN stimulation.

One of the biological actions of IFNs is the antiviral activity, which involves the production and the expression of more than 200 proteins in cells (Sadler and Williams, 2008).

The role of all these proteins is unknown, although the expression of some of them is likely to be implicated directly in the antiviral action. In particular, the protein kinase R (PKR), the 2'-5' oligoadenylate synthetase (2'-5'-OAS), and the protein Mx are the most characterized members of this class of proteins (Arnaud, 2002).

1.6.1 The dsRNA-dependent protein kinase (PKR)

The dsRNA-dependent protein kinase (PKR) is a critical mediator of the antiproliferative and antiviral effects of IFNs. PKR was identified following the observation that IFN-treated vaccinia virus (VV)-infected cells had a translational block of viral mRNAs and that cell extracts prepared from these cells showed restricted translation of viral and cellular mRNAs (Metz *et al*., 1972; Friedman *et al* ., 1972). At that time, VV was known to produce dsRNA and dsRNA was also known to inhibit protein synthesis in animal cells or in cell free systems (Cordell-Stewart *et al* ., 1973). It was demonstrated that extracts from IFN-pretreated cells had enhanced sensitivity to inhibition by dsRNA (Kerr *et al*., 1974). These studies identified a protein with dsRNA-dependent kinase activity, now known as PKR.

PKR is a serine-threonine kinase, composed by the kinase domain shared by the other kinases that phosphorylate the α subunit of eukaryotic initiation factor 2 (eIF-2α) and two dsRNA binding domains that regulate its activity. PKR is one of the four mammalian kinases (the others are GCN2, PERK, and HRI) that phosphorylate eIF-2α in response to stress signals, mainly as a result of viral infections (Barber, 2001; Dever, 2002; Harding *et al*., 2000; Williams, 1999). PKR autophosphorylation represents the activation reaction and leads to phosphorylation of eIF-2α at Ser^{51} . The phosphorylation prevents the recycling of eIF-2α that is required for ongoing translation, leading to general inhibition of translation.

In addition to the kinase domain shared by the other eIF-2α kinases, PKR also has a dsRNA-binding domain that regulates its activity. As a consequence of dsRNA accumulation in infected cells, PKR-triggered eIF-2α phosphorylation also inhibits translation of viral mRNA (Balachandran *et al*., 2000; Lee et al.,1993; Stojdl *et al*., 2000). This constitutes the basic mechanism by which PKR exerts its antiviral activity (Fig. 6).

PKR activation regulates translational and transcriptional pathways (eIF-2a and NF-kB-dependent) resulting in the specific expression of selected proteins (Fas, p53, Bax and others) that trigger cell death by engaging with the caspase pathway.

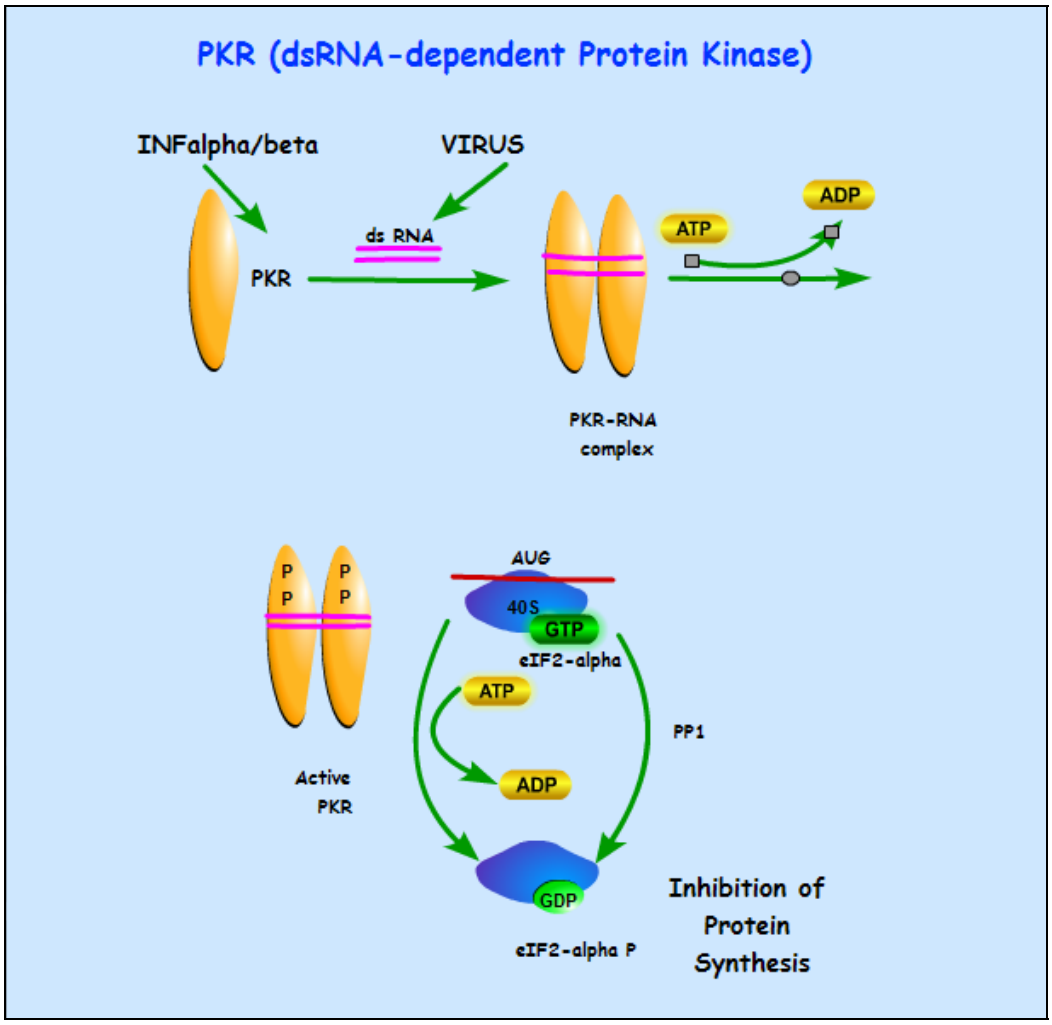


Fig. 6

The antiviral action of PKR

1.6.2 The eukaryotic initiation factor-2

In mammals, eIF-2 promotes Met-tRNAi delivery to the 40S ribosome to initiate polypeptide chain synthesis. eIF-2 is composed of three subunits (α, β and γ), it binds the Met-tRNAi in a GTP-dependent manner to form the ternary complex, which joins the 40S subunit (Hershey, 1991). Once Met-tRNAi is delivered, eIF-2 is released from the initiation complex. Inactive eIF-2–GDP complexes are continuously regenerated by GDP-to-GTP exchange in a process catalyzed by the GTP exchange

factor eIF-2B. eIF-2 activity is regulated by phosphorylation at Ser^{51} of its α subunit. As a consequence of eIF-2α phosphorylation, eIF-2 affinity for eIF-2B increases up to 100-fold, leading to competitive inhibition of eIF-2B and the resulting inhibition of translation initiation (Sudhakar *et al*., 2000) (Fig. 6).

1.6.3 2′-5′-Oligoadenylate synthetase (2'-5'-OAS)

The antiviral OAS/RNaseL system is an innate IFN-induced immunity pathway that responds to a pathogen-associated molecules to cause degradation of viral and cellular RNAs, thereby blocking viral infection (Fig. 7).

The pathogen-associated molecule is dsRNA, a type of nonself-RNA produced during infections by both RNA and DNA viruses.

Viral dsRNAs include replicative intermediates of ssRNA, viruses, viral dsRNA genomes, annealed viral RNAs of opposite polarities, and stem structures in otherwise viral ssRNAs.

2'-5'-OAS, when activated by dsRNAs, polymerizes ATP into 2'-5'-linked oligomers of adenosine, referred to as 2'-5' oligoadenylate (2'-5'-OA). The function of 2'-5'-OA is to activate a latent endoribonuclease L (RNaseL), responsible for degradation of viral and cellular RNAs. In humans four 2'-5'-OAS genes, named OAS1, OAS2, OAS3 and OASL (OAS-like), have been identified in the chromosome 12 (chromosome 5 in mice). OAS1 encodes two proteins of 40 and 46 kDa, that differ at their carboxy-termini by 18 and 54 amino acids, respectively, have one catalytic domain and form tetramers. The OAS2 gene encodes two proteins of 69 and 71 kDa which have two catalytic domains and form dimers. The OAS3 protein has three catalytic domains

and is a monomer of 100 kDa. The OASL gene produces two proteins of 30 and 59 kDa.

The 2′-5-′OA-dependent RNaseL is expressed as a 80 kDa protein with two kinase-like domains. The enzyme is constitutively expressed as an inactive monomer. Autoinhibition of the enzyme is relieved upon binding of 2′-5′-OA to the ankyrin repeats, and subsequent homodimerization. The active dimeric enzyme then degrades ssRNA . Due to their common action via RNaseL, the antiviral function of the OAS proteins has been investigated using RNaseL-deficient mice. These mice show increased susceptibility to RNA viruses from the Picornaviridae, Reoviridae, Togaviridae, Paramyxoviridae, Orthomyxoviridae, Flaviviridae and Retroviridae families. An antiviral role for RNaseL against DNA viruses is less directly established, although, as these viruses produce dsRNA replicative intermediates, they can induce 2′-5′-oligomers.

2’-5’-OAS activity is significantly induced at the acute stage of viral infections, likely as a consequence of virus-induced production of endogenous IFN-α (Read *et al*., 1985; Schattner *et al*., 1982; Zachoval *et al*., 1990), and it usually returns to basal levels when infection resolves.

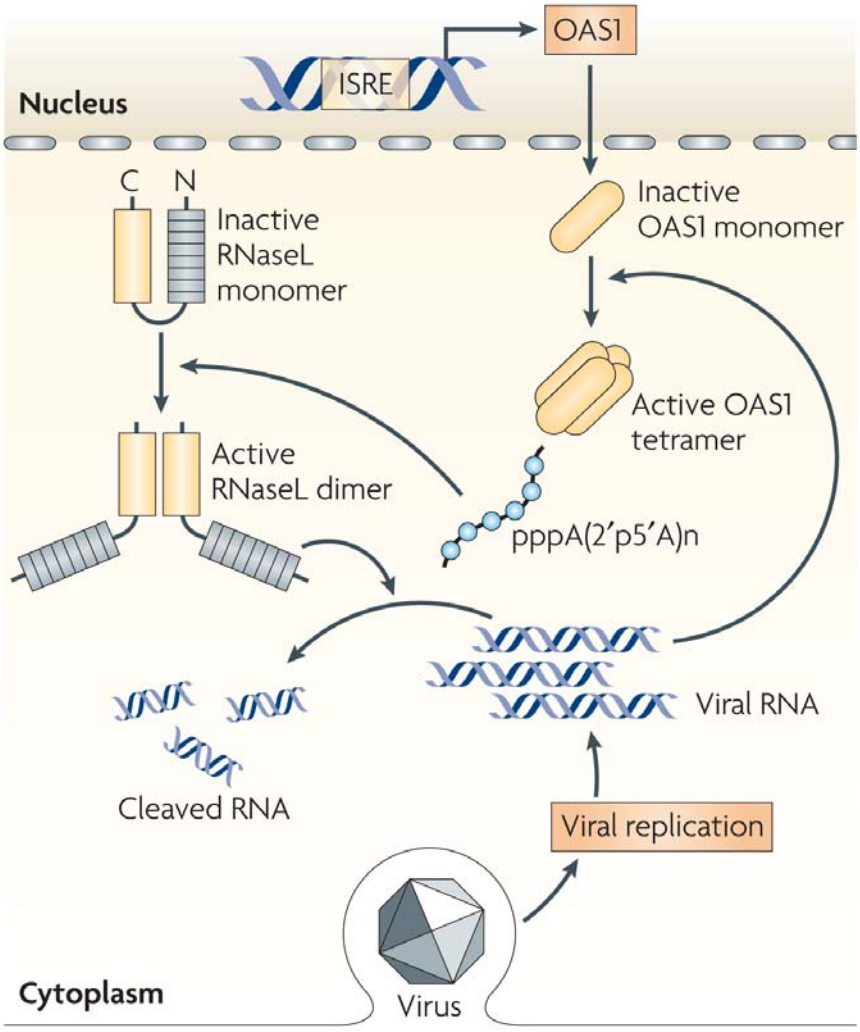


Fig. 7

The OAS-RNaseL antiviral pathway
from: Sadler and Williams, 2008

1.6.4 The myxovirus (Mx) resistance proteins

Other IFN-induced antiviral proteins are the Mx GTPases, MxA and MxB in humans and Mx1 and Mx2 in mice. The two human Mx proteins are encoded on chromosome 21, in a region syngenic to the Mx region on mouse chromosome 16 (Aebi *et al*.,

1989; Horisberger *et al*., 1988). The human proteins are cytoplasmic, as is the murine Mx2, whereas the murine Mx1 is nuclear. This differential distribution may allow each protein to target viruses that replicate in either cell compartment (Haller *et al*., 1995). Only human MxA has demonstrated antiviral activity, and this is directed against both nuclear and cytosolic viruses.

The Mx proteins have a large (relative to many GTPases) amino-terminal GTPase domain, a central interacting domain (CID) and a carboxy-terminal leucine zipper (LZ). Both the CID and LZ domains are required to recognize target viral structures. The main viral target appears to be viral nucleocapsid-like structures (Kochs *et a*l., 1999). By virtue of their location at the smooth endoplasmic reticulum, Mx proteins can effectively control exocytotic events and modulate vesicle trafficking to trap essential viral components, thereby preventing viral replication at early time points (Accola *et al*., 2002) (Fig. 8).

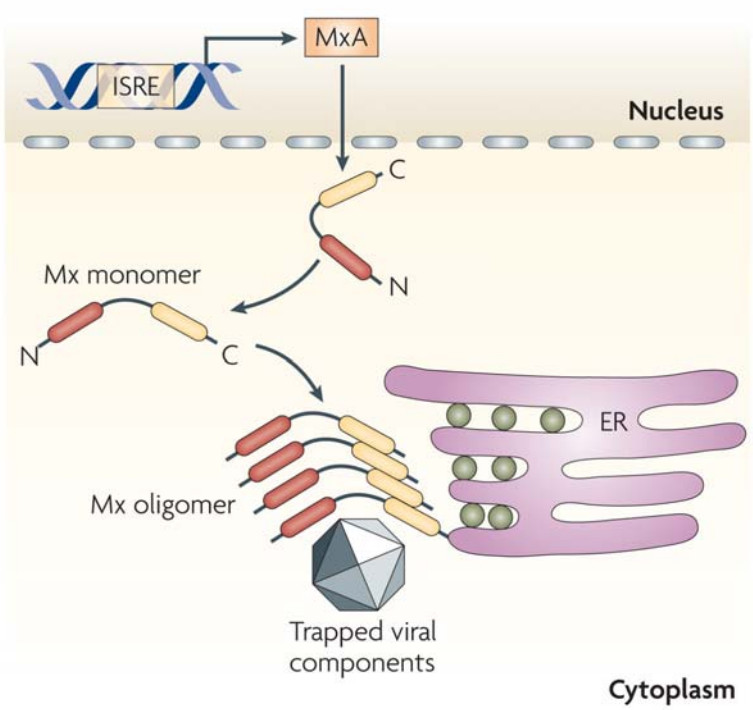


Fig. 8

Mechanism of action of the antiviral protein MxA
from: Sadler and Williams, 2008

1.7 Clinical use of IFNs

The biomedical interest in IFNs is linked to their therapeutic efficacy against viral infections (in particular against HCV and HBV), different types of cancer and neuroinflammatory diseases, particularly multiple sclerosis (Borden *et al*., 2007). Currently, IFNs are mainly employed as a standard therapy for hairy cell leukemia, metastasizing renal carcinoma and AIDS-associated angiogenic tumors of mixed cellularity known as Kaposi sarcomas. Three kinds of human IFNs (α, β, and γ), have been approved for clinical use by the Food and Drug Administration.

Protein	Year	Clinical use
Interferon-α2a	1988	Melanoma; Kaposi sarcoma; non-Hodgkin lymphomas, leukemia; Chronic hepatitis B e C
Interferon-α2b	1986	
PEG-interferon-α2a	2002	Chronic hepatitis C (± ribavirin)
PEG-interferon-α2b	2001	
Interferon-α consensus	1997	Chronic hepatitis C
Interferon-γ	1990	Osteopetrosis, granulomatosis
Interferon-β1a	1996	Multiple sclerosis (relapsing/remitting form)
Interferon-β1b	1993	

Table 3

Clinical use of IFNs

IFN-α species are most widely used for diseases of known viral origin, such as hepatitis C and hepatitis B. The very first treatment shown to be effective against HCV involved systematic administration of INF-α. Through the years, HCV treatment has improved with the development of modified pegylated-interferon (PEG-IFN), which has longer biological half-life. Currently, PEG-IFN and ribavirin combination is the standard treatment for HCV. Unfortunately, both these compounds are toxic and their administration causes a number of side effects. Moreover, the cost of therapy is very high and several months of therapy are required for eradication of chronic HCV infection, which is considered one of the major causes of chronic liver disease and one of the major causes of death in the U.S.A.

IFN-β1b (Betaseron) and IFN-β1a (Avonex, Rebif), are approved for the treatment of relapsing-remitting multiple sclerosis (MS). MS is a chronic, potentially debilitating disease of unknown etiology that affects the brain and the spinal cord. Treatment of MS with IFN-β was initiated based on the immunomodulatory properties of the IFNs. Well-controlled studies have shown that treatment with IFN-β results in a reduction of the annual rate of relapses of MS. Initiation of IFN-β treatment with the first instance of demyelination in MS appears to be a justified therapeutic intervention (Jacobs *et al*., 2000)

With regard to IFN-γ, it is currently used in therapy for chronic granulomatous disease and skin lesions.

1.8 Type I IFN side effects

The therapeutic use of type I IFNs causes a number of peripheral and central adverse effects, which compromise tolerability and often limit the high dosage required to control cancer or viral disease (Vial *et al*., 2000; Raison *et al*., 2005).

The number of different adverse events reported during therapy with IFNs is impressive (Fattovich *et al*., 1996). The identification of etiopathogenetic link with IFN-α administration may be difficult, especially if one considers that IFN-α must be administered continuously for several months: in the case of chronic hepatitis B for 48-week, while in the case of chronic hepatitis C, the length of therapy may vary between 12 and 72 weeks, depending on the pattern of response. The most frequent effects reported by patients treated with IFNs are referred to as a typical flu-like syndrome with chills, fever, arthralgias, myalgias, rigours, headache, fatigue, weakness, and nonspecific digestive complaints like nausea, abdominal pain and

constipation. Altogether, flu-like symptoms are reported by almost all patients under therapy, are more intense after the first injections and tend to decrease after 4–6 weeks from start. The reason why IFNs provoke fever is not clear. It has been postulated that IFNs may be directly pyrogenic in the hypothalamus, acting to increase prostaglandin production.

The most important cause of drug withdrawal is the haematological toxicity, in particular neutropenia, due to the myelosuppressive effect. Moreover, myelosuppression-related anaemia is consistently observed after administration of IFNs. However, this effect is less worrying compared to the haemolytic anaemia caused by ribavirin, which is almost invariably administered concomitantly with IFN-α. IFNs treatment is also associated with the development of hypo- or hyperthyroidism (Fattovich *et al*., 1996). Up to 6% of treated subjects will develop thyroid abnormalities (Watanabe *et al*., 1994; Deutsch *et al*., 1997).

1.9 Neurotoxicity of type I IFNs

The most serious and dangerous IFN adverse effects involving the central nervous system are the neuropsychiatric disturbances. The spectrum of psychiatric side effects includes irritability, anxiety, depression, psychosis and suicide. Depression has been reported to occur in 7-35% of patients depending on the duration of IFN treatment (Vial *et al*., 2000; Raison *et al*., 2005).

Although the neuropsychiatric effects most often subside after IFN dosage reduction or discontinuation, there are reports that IFN neurotoxicity may persist for months or years after IFN withdrawal (Meyers *et al*., 1991).

Animal models have been used to study whether IFNs may have the potential to induce symptoms or changes associated with depression. Laboratory animal studies have been performed administering IFN peripherally or centrally in rodents. These studies have demonstrated a dose-dependent increase in the immobility time in the forced swimming test, (Makino *et al*., 1998, 2000), an increase in corticotrophin releasing hormone (CRH) release in the amygdala and hypothalamus (Raber *et al*., 1997), and a decrease in the brain dopamine and serotonin concentrations (Shuto *et al*., 1997; Kamata *et al*., 2000). In rhesus monkeys, 4 weeks of IFN therapy increased plasma concentration of adrenocorticotropic hormone and cortisol, and induced depressive-anxiety-like behaviors that resemble the human pathology (Felger *et al*., 2007).

The pathogenesis of the neuropsychiatric side effects is still not completely understood.

It has been suggested that IFNs may affect different neurochemical pathways putatively involved in the etiology of depression, including the serotonergic system, the hypothalamus-pituitary-adrenal (HPA) axis, the hypothalamus-pituitary-thyroid axis, and the cytokine network.

IFNs are potent stimulators of the HPA axis, increasing the release of CRH and cortisol (Wichers and Maes, 2002; Felger *et al*., 2007), and evidence exists for a hyperactive HPA axis in depression (van West and Maes, 1999).

Type I IFNs activate intracellular signaling pathways that may induce or inhibit the expression, or modify the effects of several cytokines, including IFN-γ, IL- 1, IL-2, IL-6, IL-8, and TNF-α (Taylor and Grossberg, 1998).

Moreover IFNs can affect serotonin metabolism directly and/or indirectly by inducing the enzyme indoleamine 2,3-dioxygenase (IDO), which leads to a peripheral depletion of tryptophan and its conversion into kynurenine (Taylor and Feng, 1991). The kynurenine metabolites 3-hydroxy-kynurenine (3-OH-KYN) and quinolinic acid (QUIN) are neurotoxic, as demonstrated in animals and in vitro experiments (Okuda *et al*., 1998). Increased production of 3-OH-KYN and QUIN may also contribute to neuronal damage in cognitive decline of aging, infections of the central nervous system, ischemia, hypoxia at birth, traumatic injury and epilepsy. In addition, they may play a role in the development of psychiatric diseases such as anxiety, depression and schizophrenia (Stone *et al*., 2001; Reynolds *et al*., 1988; Ogawa *et al*., 1992)

There is also evidence that peripherally administered IFNs can enter the brain and exert direct actions on brain structures (Pan *et al*., 1997; Thorne *et al*., 2008). In the brain, type I IFNs have been reported to cause alterations of opioid, monoamine and glutamate transmission and to induce cytokine and chemokine release from microglia (Kim *et al*., 2002; Dafny and Yang, 2005). It has been suggested that glia-derived factors may play a role in the alterations of monoamine neurotransmission and induction of neuropsychiatric side effects (Raison *et al*., 2009).

However, an additional possibility is that type I IFNs may exert toxic effects directly on neuronal cells. Gene chip analysis of RNA from primary brain cell culture treated with IFN-α indicated that neurons are very responsive target cells for IFNs (Wang and Campbell, 2005).

At the present, little is known on the expression of functional type I IFN receptors in human neuronal cells and on the ability of these cytokines to affect neuronal cell

viability by regulating signaling pathways governing cell survival and apoptosis (Fig. 9).

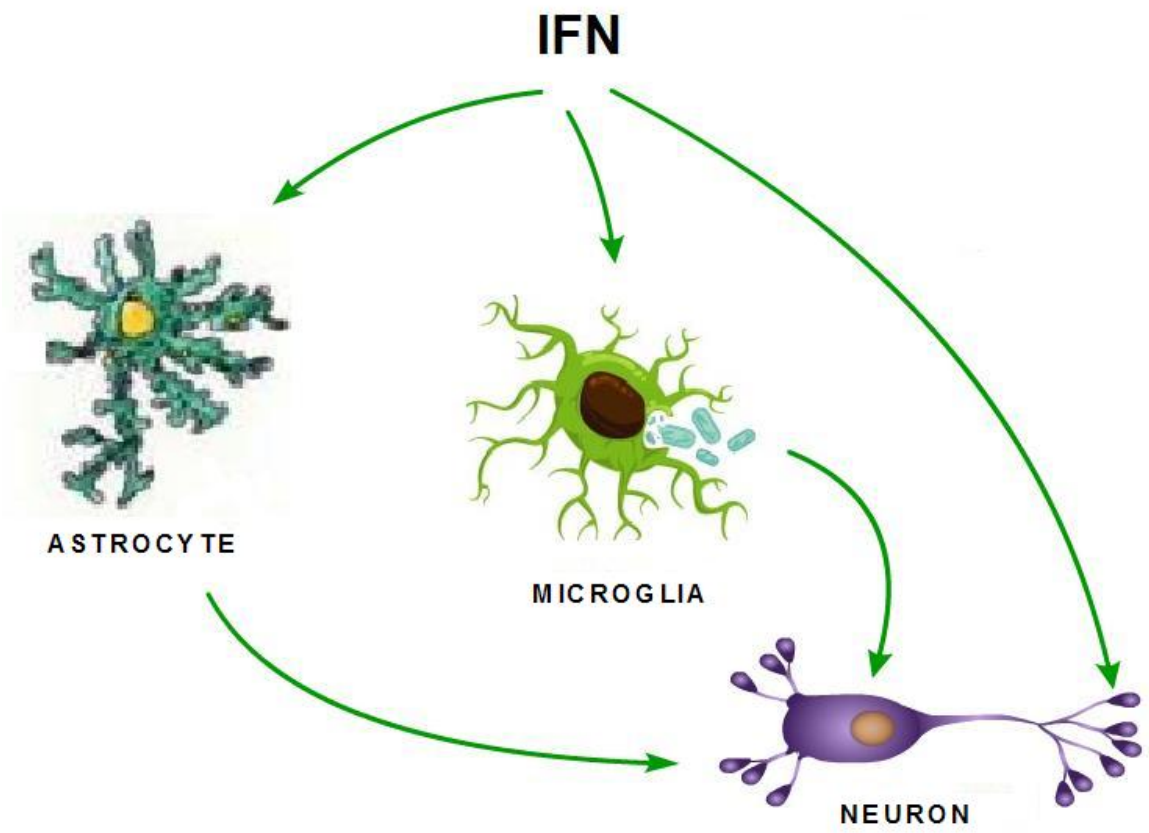


Fig. 9

Possible cellular targets of type I IFNs in the Central Nervous System

2. RESEARCH SECTION

2.1 Aims

The aims of the study are: a) to investigate the expression and functional activity of IFN receptors in neuronal cells and their role in neuronal survival; b) to assess whether neurons represent a direct target of IFN neurotoxicity; c) to identify pharmacological or genetic approaches capable of preventing or counteracting the neuropsychiatric side effects elicited by these cytokines.

The study involved:

a) the preparation of primary neuronal cell cultures and the assessment of their sensitivity to IFN action;

b) the identification of a suitable neuronal cell line to study the direct neurotoxic action of IFNs;

b) the analysis of the changes in cellular protein expression and phosphorylation induced by IFN treatment;

c) the evaluation of the alterations in neuronal survival and synaptic plasticity following long-term incubation with IFNs.

2.2 Materials and Methods

2.2.1 Materials

Recombinant human IFN-β1a, IFN-α2b and TNF-α, and mouse IFN-β were obtained from ProSpec-Tany TechnoGene Ltd (Rehovot, Israel). Recombinant human IFN-β1b and IFN-α2a were obtained from Schering (Milano, Italy) and Roche (Milano, Italy), respectively. Recombinant insulin–like growth factor I (IGF-I), all-trans-retinoic acid

(RA), phorbol 12-myristate 13-acetate (PMA) AR A014418 and actinomycin D were from Sigma-Life Science (St. Louis, MO, USA). Pyridone 6 (P6), PKR inhibitor and neutralizing mouse monoclonal anti-IFNAR2 antibody were from Calbiochem (San Diego, CA, USA). Normal mouse IgG was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The cell-permeable irreversible pan-caspase inhibitor Z-VAD-FMK was obtained from Biovision Research Products (Mountain View, CA, USA). Recombinant human brain-derived neurotrophic factor (BDNF) was obtained from Alomone Labs (Jerusalem, Israel). SB203580 and SB216763 were from Tocris (Bristol, UK) and Ascent Scientific (Bristol, UK), respectively.

2.2.2 Preparation of primary cultures of mouse neuronal cells.

Primary cultures of neurons derived from the cerebral cortex and subcortical structures (hippocampus and basal ganglia) were prepared from 1 day-old mice by using trypsin digestion as previously described (Olianas *et al*., 2008). Dissociated cells were collected by centrifugation and resuspended in Neurobasal A medium containing B27 serum-free supplement, 0.5 mM L-glutamine, 50 µM β-mercaptoethanol and penicillin-streptomycin (Invitrogen). Cells were plated on either glass coverslips (Electron Microscopy Sciences, Fort Washington, PA, USA) or 12-well plates pre-coated with 0.01% poly-L-lysine (Sigma) at a density of 1.5×10^4 and 0.5×10^6 cells/cm^2, respectively. The medium was removed 2 h later. Cultures were used five days after plating and contained 90 to 95% neuronal cells as assessed by immunofluorescence staining for neuron-specific enolase (NSE), neurofilament 160/200 (NF160/200), which recognizes NF-M and NF-H (~ 160 and 200 kDa), and glial fibrillary acidic protein. Three separate culture preparations were used.

Experiments were performed according to the principles of laboratory animal care (Law on animal experiments in Italy, D.L. 116/92).

2.2.3 Cell lines and culture conditions

SH-SY5Y cells were obtained from the European Cell Culture Collection (Salisbury, UK) and grown in Ham's F12/MEM medium (1:1) supplemented with 2 mM L-glutamine,1 % non-essential amino acids, 10% fetal calf serum (FCS) and 100 U/ml penicillin-100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. Cells were used up to the 16th passage. Cells were differentiated by exposure to 10 µM RA for 6 days (Pahlman *et al.*, 1984) or by sequential treatment with 10 µM RA for two days and 100 nM PMA for two additional days (Pesgraves *et al*., 2004).

HeLa cells (American Type Culture Collection, Manassas, VA, USA) were grown in Minimal Essential Medium (Invitrogen) supplemented with 10% FCS and L-glutamine at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

2.2.4 Cell treatment, immunoprecipitation and Western blot analysis

Cells were washed with phosphate buffered saline (PBS) and incubated in medium containing 1% FCS. Cells were stimulated with IFNs as indicated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Inhibitors were dissolved in dimethyl sulfoxide (DMSO) and added before IFNs as indicated. The final concentration of DMSO in the incubation medium was < 0.5%. Control samples received an equal amount of vehicle. When the effect of mouse monoclonal anti-IFNAR2 antibody (Calbiochem) was examined, cells were pre-treated for 1 h with the indicated

antibody concentrations and then stimulated with IFN. Control samples were pre-treated with equal concentrations of normal mouse IgG.

Cells were washed and incubated with ice-cold cell lysis buffer containing PBS, 0.1 % sodium dodecyl sulphate (SDS), 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 2 mM EDTA, 2 mM EGTA, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 20 nM okadaic acid, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 % phosphatase inhibitor cocktail 1 and 1 % protease inhibitor cocktail (Sigma). The samples were sonicated for 5 s in ice-bath and aliquots of cell extracts were taken for protein determination by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

For immunoprecipitation of insulin receptor substrate-1 (IRS-1), pre-cleared cell extracts were incubated with agarose-conjugated rabbit polyclonal anti-IRS-1 antibody (Santa Cruz) overnight at 4 °C with continuous rocking. The beads were then washed four times with lysis buffer and mixed with electrophoresis sample buffer.

Cell proteins were separated by SDS-polyacrylamide gel (SDS-PAGE) and electrophoretically transferred to nitrocellulose or polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). The transfer efficiency was controlled by gel staining and by using pre-stained protein standards (Santa Cruz). Membranes were blocked by incubation in 20 mM Tris-HCl, 137 mM NaCl and 0.1 % Tween-20 (pH 7.6) (TBS-T buffer) containing either 5 % BSA or 5 % non-fat dry milk (Santa Cruz) for 1 h. After washing with TBS-T buffer, the membranes were incubated overnight at 4 °C with one of the following primary antibodies: mouse monoclonal anti-phospho-(Tyr701)-STAT1, anti-phospho-(Tyr694)-STAT5 (detecting

both STAT5a and STAT5b), anti-pan STAT5a/b and anti-STAT3 (Zymed Laboratories, San Francisco, CA, USA); rabbit polyclonal anti-phospho-(Tyr1054/1055)-Tyk2, anti-Tyk2, anti-procaspase-8, anti-cleaved caspase-9, anti-procaspase-9, anti-cleaved caspase-7, anti-procaspase-7, anti-cleaved caspase-3, anti-procaspase-3, anti-cleaved poly-(ADP ribose) polymerase (PARP), anti-PARP, anti-PKR, anti-phospho-(Ser51)-eukaryotic initiation factor 2α (eIF2α), anti-eIF2α, anti-phospho-(Ser473)-Akt and anti-phospho-(Ser9)-glycogen synthase kinase (GSK)-3β, anti phospho-(Thr180/Tyr182)-p38 MAP Kinase and rabbit polyclonal anti-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA); rabbit polyclonal anti-phospho-(Thr308)-Akt (GenWay Biotech. Inc., San Diego, CA, USA); rabbit anti-phospho(Thr202/Tyr204)extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) (Neuromics, Northfield, MN); mouse monoclonal anti-phospho-(Tyr705)-STAT3, anti-phospho-tyrosine (PY99), rabbit polyclonal anti-STAT1, anti-Akt1/2/3, anti-GSK-3β, and anti-IRS-1 (Santa Cruz); mouse anti-p38 MAPK (StressMarq Biosciences Inc., Victoria, Canada); mouse monoclonal anti-tyrosine hydroxylase (TH) and rabbit polyclonal anti-actin (Sigma); rabbit polyclonal anti-phospho-(Ser31)-TH (Chemicon-Millipore, Temecula, CA, USA),

Thereafter, the membranes were washed and incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz). Immunoreactive bands were detected by using ECL-Plus reagent and ECL Hyperfilm (Amersham). The size of immunoreactive bands was determined by using molecular weight standards detected with an ECL suitable antibody (Santa Cruz). Band densities were determined by densitometric analysis using Image Scanner III (GE Healthcare, Milan, Italy) and NIH ImageJ software (US National Institutes of Health,

Bethesda, MA, USA). The optical density of phosphoprotein bands was normalized to the density of the corresponding total protein or actin band to yield the relative optical density (ROD) value.

2.2.5 Cell viability assay and morphological analysis

Cells grown on glass coverslips (Electron Microscopy Sciences, Fort Washington, PA, USA) pre-coated with 0.01% poly-L-lysine (Sigma) were treated with either IFN-β (30 ng/ml) or vehicle for 24 h. Thereafter, cells were incubated with 1 μg/ml of propidium iodide (PI) (Sigma) for 1 h, washed, fixed in 4 % paraformaldehyde and incubated for with 0.1 μg/ml 4',6-diamidino-2phenylindole dihydrochloride (DAPI) (Sigma). Coverslips were mounted with Gel Mount aqueous mounting medium (Sigma) and cells were examined by fluorescence microscopy by using an Olympus IX71 microscope. Images were captured over randomly selected fields using a 20 X objective lens and Olympus Fwiew II digital camera at constant settings and analyzed with the software Cell P (Olympus). PI positive cell nuclei were counted and their number expressed as percent of total nuclei. The number of fragmented and condensed nuclei was determined by DAPI staining. Three coverslips were used per experimental group and approximately 200 cells were counted in four random fields of each sample. For morphological examination, phase-contrast images were obtained from control and IFN-treated cells in randomly selected fields. Three separate preparations were analyzed by an investigator unaware of the experimental protocol.

2.2.6 Immunofluorescence analysis.

For immunofluorescence analysis of phospho-STAT1, primary cultures of mouse neuronal cells or SH-SY5Y cells grown on pre-coated glass coverslips were treated for 30 min with either vehicle, mouse or human IFN-β.(30 ng/ml). For immunofluorescence analysis of cleaved caspase-3, cells were treated for 6 h with either vehicle or P6 (2 μM) and then incubated for 24 h with either vehicle or mouse IFN-β (30 ng/ml).

Cells were washed, fixed, permeabilized with 0.2 % Triton X-100 and blocked with 3 % BSA and 1 % normal goat serum. Cells were then incubated overnight at 4 °C with either rabbit polyclonal anti-NSE (1:300) (Biomol, Hamburg, Germany) and mouse monoclonal anti-phospho-(Tyr701)-STAT1(1:200) or mouse monoclonal anti-NF160/200 (1:500) (Sigma) and rabbit anti-cleaved caspase-3 (1:200). The cells were washed and incubated with a mixture of Alexa 488-conjugated goat anti-rabbit IgG (1: 3000) and Alexa 594-conjugated goat anti-mouse IgG (1:4000). After staining cell nuclei with DAPI, images were acquired over randomly selected fields using a 40X objective lens and analyzed with Cell P software. Cells were deemed to be positive for cleaved caspase-3 if the average pixel intensity of the green fluorescence was equal or above a threshold value corresponding to one standard deviation above the average pixel intensity of the cells in control samples. The percent of positive cells was calculated as the number of positive cells / total number of nuclei stained by DAPI X 100. Three separate culture preparations were analyzed by an investigator unaware of the treatment.

For neuritogenesis measurements, SH-SY5Y cells were differentiated in pre-coated glass coverslips in the presence of 10μM RA for 6 days. Thereafter, cells were grown

in a medium containing 1% FCS in the presence of the test agents for 48 h. Thereafter, cells were washed, fixed in 4 % paraformaldehyde, permeabilized with 0.2 % Triton X-100 and blocked for 1 h at room temperature with 3% BSA and 1% nonimmune serum appropriate for the secondary antibody. Cells were then incubated overnight at 4 °C with anti-NF160/200 (1:500) followed by Alexa 594–conjugated goat anti-mouse IgG (1:4000). After extensive washing, cells were incubated with fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma) for 1 h and the cell nuclei were stained with 0.1 µg/ml DAPI. Coverslips were washed, covered with Gel Mount and examined on an Olympus IX71 fluorescence microscope. The cell nuclei and neurites present in 12 randomly selected fields for each sample were counted. The ratio of neurites to cell nuclei was then determined for each experimental sample. Assays were run in duplicates.

2.2.7 RNA interference

Human STAT1-p84/p91 small interfering RNA (siRNA) (sc-44123) and control siRNA were obtained from Santa Cruz. SH-SY5Y cells were transfected with siRNA oligonucleotides (50 pmol/ml) using Lipofectamine 2000 reagent (Invitrogen).

2.2.8 Terminal transferase dUTP nick end-labeling assay (TUNEL assay)

SH-SY5Y cells grown on glass coverslips were incubated with either vehicle (control) or IFN-β (30 ng/ml) for 24 h. The pan-caspase inhibitor Z-VAD-FMK was added at the concentration of 35 µM 3 h after IFN. When the effect of constitutively active Akt was examined, cells were transiently transfected with the construct myr-Akt1

(Millipore-Upstate, Temecula, CA, USA). Control cells were transfected with equal amount (0.2 µg) of empty vector. Cells were analyzed 48 h following transfection.

Cells were fixed in ice-cold 4 % paraformaldehyde and permeabilized with 0.2 % Triton X-100. TUNEL assay was performed using the DeadEnd fluorimetric TUNEL system (Promega, Madison, WI, USA), according to the manufacturer's instructions. Images were captured over randomly selected fields and analyzed with Cell P software. For each cell examined, the average pixel intensity of the green fluorescence in the nucleus was determined and corrected for the fluorescence intensity of an adjacent area, which was considered as background value. Cells were deemed to be positive if the average pixel intensity was equal or above a threshold value corresponding to one standard deviation above the average pixel intensity of the cells in control samples. The percent of positive cells was calculated as the number of positive cells / total number of nuclei stained by DAPI X 100. At least three separate culture preparations were analyzed by an investigator unaware of the treatment.

2.2.9 Akt activity assay

Akt activity was assayed by using a non-radioactive assay kit obtained from Cell Signaling Technology. Cells were treated with either vehicle or IFN-β (30 ng/ml) for 24 h, washed with PBS and lysed in ice-cold cell lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1µg/ml leupeptin and 1 mM PMSF. Samples were centrifuged and supernatants assayed for protein content. Aliquots containing equal amount of protein (0.8-1.0 mg) were added

to agarose crosslinked to mouse monoclonal anti-Akt antibody and incubated overnight at 4 °C with continuous rocking. The beads were then washed with cell lysis buffer and with kinase assay buffer containing 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate and 10 mM $MgCl_2$. Thereafter, the beads were resuspended in kinase assay buffer supplemented with 0.2 mM ATP and 20 µg/ml of GSK-3α/β crosstide (corresponding to residues surrounding Ser21/9 and expressed as GST-fusion protein) and the samples were incubated for 30 min at 30 °C. The reaction was stopped by the addition of sample buffer, the samples were heated at 100 °C and analyzed by Western blot using a rabbit polyclonal antibody against phospho-Ser21/9-GSK-3α/β. The blot was reprobed with an antibody against Akt to control enzyme immunoprecipitation and the optical density of the phospho-Ser21/9-GSK-3α/β band was normalized to the density of the corresponding Akt band. Three separate cell preparations were examined.

2.2.10 Preparation of cellular fractions

Cells were grown in 100 mm Petri dishes, incubated in medium supplemented with 1 % FCS and treated for 24 h with either vehicle or 30 ng/ml IFN-β1b. Cells were harvested by scraping in ice-cold PBS, centrifuged at 600 g for 10 min at 4 °C and resuspended in ice-cold homogenization buffer (100 mM Tris/MOPS, 0.2 M sucrose, 1 mM EGTA, pH 7.4, supplemented with 10 mM sodium fluoride, 1 mM PMSF, 0.1 % phosphatase inhibitor cocktail 1 and 1 % protease inhibitor cocktail). Cells were homogenized by using a Teflon/glass tissue grinder operated at 800 rpm (20 strokes) at ice-bath temperature, the homogenate was centrifuged at 1000 g for 10 min and

the supernatant centrifuged at 12,000 g for 15 min at 4 °C. To examine cytochrome C release, the supernatant was used as cytosolic fraction, whereas the pellet containing the mitochondria was washed and resuspended in 100 µl of homogenization buffer. Aliquots of each fraction containing an equal amount of protein were mixed with sample buffer and loaded on 12 % SDS-PAGE.

2.2.11 Transfection-based reporter gene assay

Cells were seeded in 12-well plates, incubated overnight and transfected with either 400 ng/well of a reporter vector containing the interferon stimulated regulatory element (ISRE) located upstream of luciferase coding sequence (pISRE-Luc, Clontech Lab., Mountain View, CA, USA) or pTA-Luc vector as negative control by using the calcium phosphate transfection protocol according to the manufacturer's instructions. Each sample was cotransfected with 2 µg of pSV-β-galactosidase control vector (Promega) as internal control. After 12-14 h the medium was removed and the cells were treated with the test agents in 0.1 % FCS medium. Forthy hours post-transfection, cells were lysed with Reporter Lysis Buffer (Promega) and aliquots of the cell extracts were assayed for luciferase activity and for β-galactosidase activity by using the Bright-Glo Luciferase assay system and the β-Galactosidase enzyme assay system (Promega), respectively. The luciferase activity of each sample was normalized on the basis of the β-galactosidase activity. The experiments were carried out in triplicate.

2.2.12 Statistical analysis

Results are reported as mean ± standard error of the mean (SEM). Data from concentration-response curves were analysed by the program Graph Pad Prism (San Diego, CA, USA.). Statistical analysis was performed by either Student's t test or one-way ANOVA followed by Newman-Keuls *post ho*c test as appropriate.

3. RESULTS

3.1 IFN-β induces STAT phosphorylation and activates caspase-3 cleavage in primary neurons

In primary cultures of mouse neuronal cells short-term treatment (30 min) with mouse IFN-β (30 ng/ml) elicited a marked increase of STAT1 and STAT3 tyrosine phosphorylation (Fig. 10a). Immunocytochemical analysis showed that IFN-β treatment increased phospho-Tyr-STAT1 labeling in cell bodies and processes of cells positive for the neuronal marker NSE (Fig. 10b).

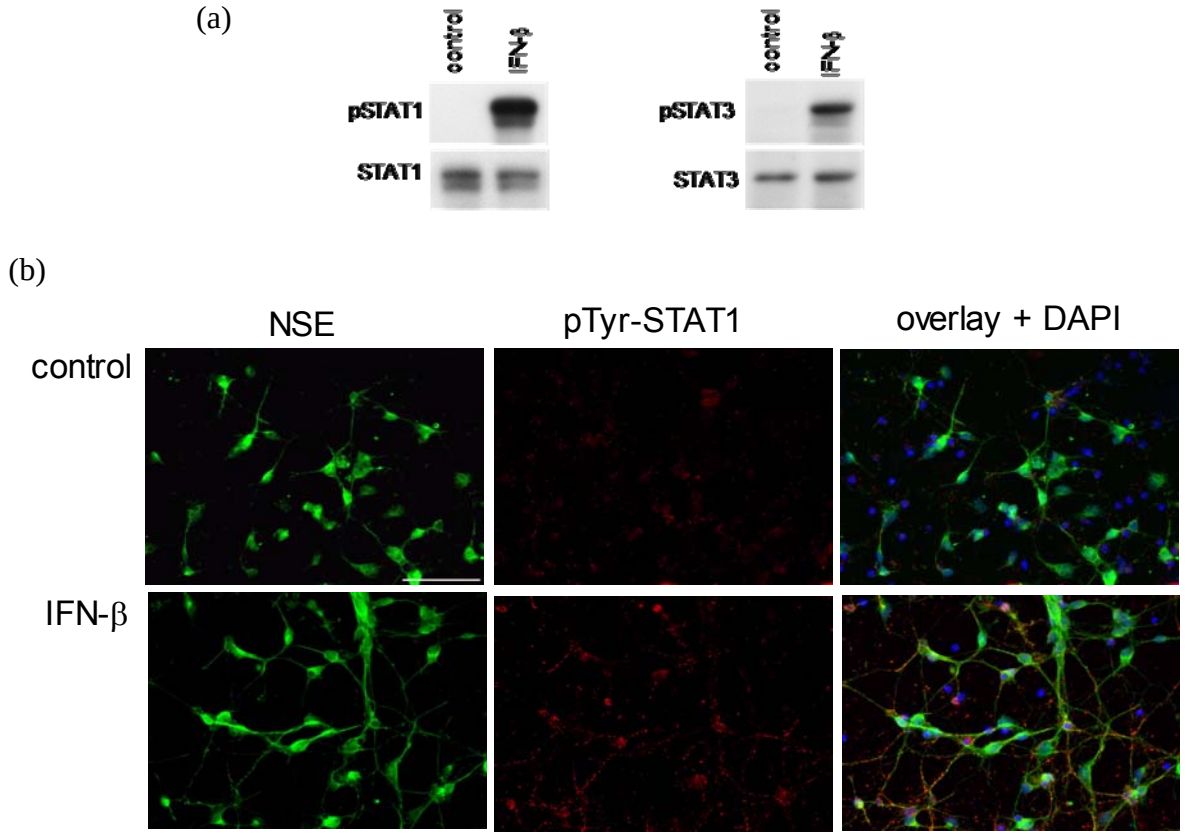


Fig. 10

IFN-β induces STAT tyrosine phosphorylation and caspase-3 activation in primary neurons**.** (a) Cells were treated for 30 min with either vehicle or mouse IFN-β (30 ng/ml). Data are representative of three similar experiments. (b) Immunocytochemical analysis of STAT1 tyrosine phosphorylation in neuronal cultures treated for 30 min with either vehicle (control) or mouse IFN-β (30 ng/ml). Cells were stained for NSE (green) and phospho-Tyr-STAT1 (p-Tyr-STAT1) (red). Nuclei were stained with DAPI. Images are representative of three separate experiments. Scale bar = 50 µm. *Modified from: Dedoni et al., 2010*

Long-term exposure (24 h) of primary neurons to mouse IFN-β (30 ng/ml) affected neuronal morphology, causing loss of neuritic processes and significantly increased the expression of cleaved caspase-3 (Fig. 11).

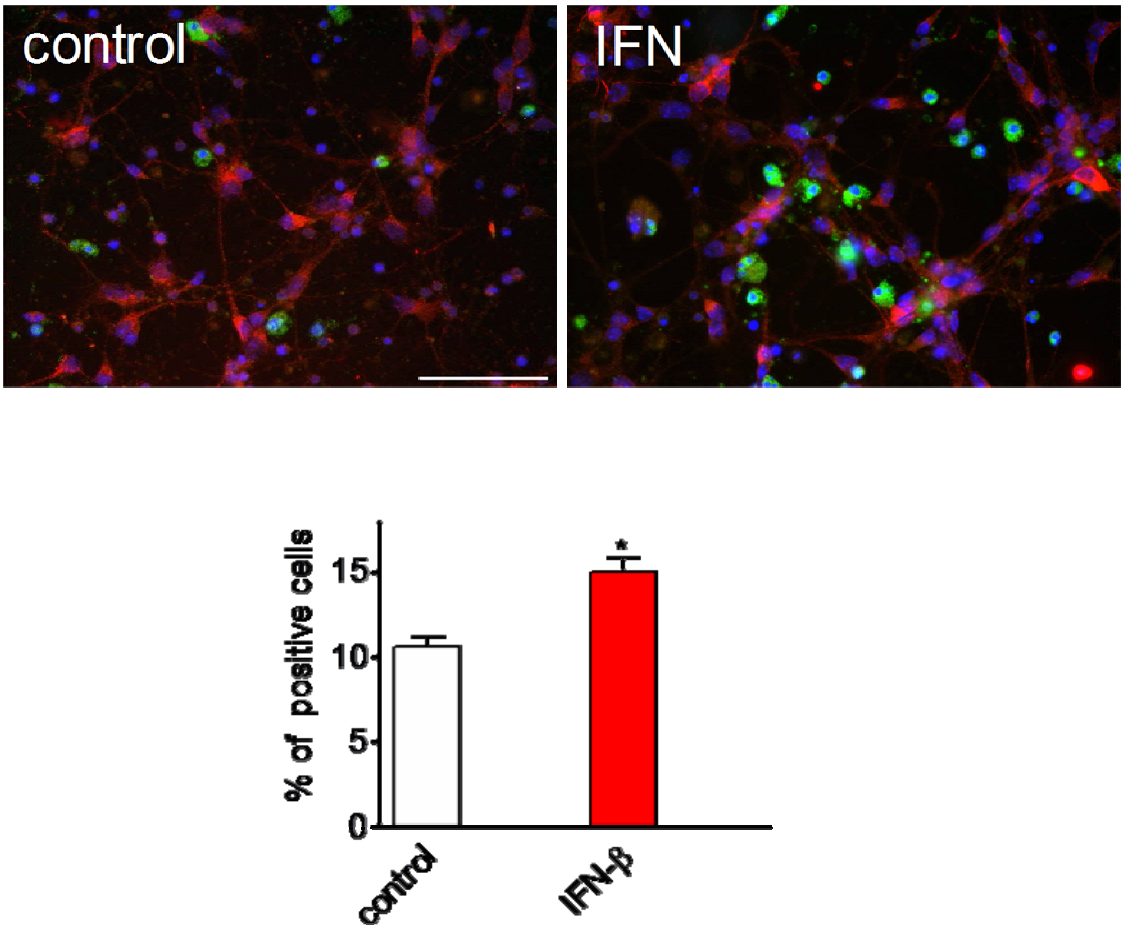


Fig. 11

Immunocytochemical analysis of cleaved caspase-3 in neuronal cultures incubated for 24 h with either vehicle or mouse IFN-β (30 ng/ml). Neuronal cells were stained for NF160/200 (red) and cleaved caspase-3 (green). Nuclei were stained with DAPI. For each treatment the percent of cells positive for cleaved caspase-3 are reported. Values are the mean ± SEM of three experiments performed in three separate culture preparations. * $p < 0.05$ vs control (vehicle + vehicle). Scale bar = 50 μm. *Modified from: Dedoni et al., 2010*

2.3.2 IFN-α and IFN-β trigger JAK-STAT signaling in SH-SY5Y cells.

Although the primary cultures of mouse cortical cells contained 90-95% neurons, it might have been possible that the observed IFN effects on neurons were mediated through the release of other cytokines from the limited population of glial cells. To gain a more compelling evidence for a direct action of type I IFN on neuronal cells, the human neuroblastoma cell line SH-SY5Y, which constitutes a relatively homogeneous population of neuron-like cells, was employed. This cell line has been widely used as a cellular model to study the intracellular mechanisms mediating the actions of drugs on human neurons (Xie *et al*., 2010).

As a first step, it was necessary to assess whether SH-SY5Y cells expressed functional type I IFN receptors. To this aim, the effects of a short-term exposure to IFN-α and IFN-β on Tyk2 and STAT tyrosine phosphorylation were investigated. As shown in Fig. 12 (a), IFN-α and IFN-β caused a rapid phosphorylation of Tyk2 on Tyr1054/1055 located in the kinase activation loop, an event required for enzyme activation. With both cytokines, Tyk2 phosphorylation reached a maximum at approximately 15 min and remained elevated for at least 30 min. Within the same time frame, IFN-α and IFN-β induced the phosphorylations of STAT1 on Tyr 701, STAT3 on Tyr705 and STAT5 on Tyr 694 (Fig. 12b-d), which are required for STAT dimerization and translocation to the nucleus (Levy and Darnell, 2002). Analysis of STAT1, STAT3 and STAT5 and their phosphorylated forms at longer IFN-β incubation times showed that STAT1 expression increased by two-fold ($p < 0.001$) at 6 h and remained up-regulated at 24 h, whereas STAT3 was modestly enhanced only at 24 h (Fig. 12e). STAT5 expression was not significantly changed at each time point investigated (result not shown). Moreover, while phospho-STAT3 was detected

at 1 h but not at longer incubation times, phospho-STAT1 was still significantly enhanced at 3, 6, and 24 h of IFN-β exposure. Phosphorylation of STAT5 was not detected at 3, 6, and 24 h (results not shown). Thus, STAT1, STAT3 and STAT5 displayed different long-term regulation by IFN-β.

Type I IFN-induced tyrosine phosphorylation of Tyk2 and STATs occurred in a concentration-dependent manner and was saturable (Fig. 13a-d). IFN-α2a, IFN-α2b, IFN-β1a and IFN-β1b stimulated STAT1 phosphorylation with EC_{50} values of 0.54 ± 0.07, 1.70 ± 0.2, 1.50 ± 0.09 and 0.40 ± 0.06 ng/ml, respectively. Similar EC_{50} values of IFN-α2a and IFN-β1b were observed for the stimulation of STAT3 phosphorylation (1.69 ± 0.08 and 1.53 ± 0.07 ng/ml, respectively), STAT5 (0.70 ± 0.10 and 0.99 ± 0.09 ng/ml, respectively) and Tyk2 phosphorylation (1.40 ± 0.06 and 1.60 ± 0.20 ng/ml, respectively). The involvement of specific type I IFN receptor was investigated by using a neutralizing antibody directed against the IFNAR2 subunit of the receptor. As shown in Fig. 13 (e), pre-incubation of the cells with the anti-IFNAR2 antibody elicited a concentration-dependent inhibition of IFN-β-stimulated STAT1 tyrosine-phosphorylation.

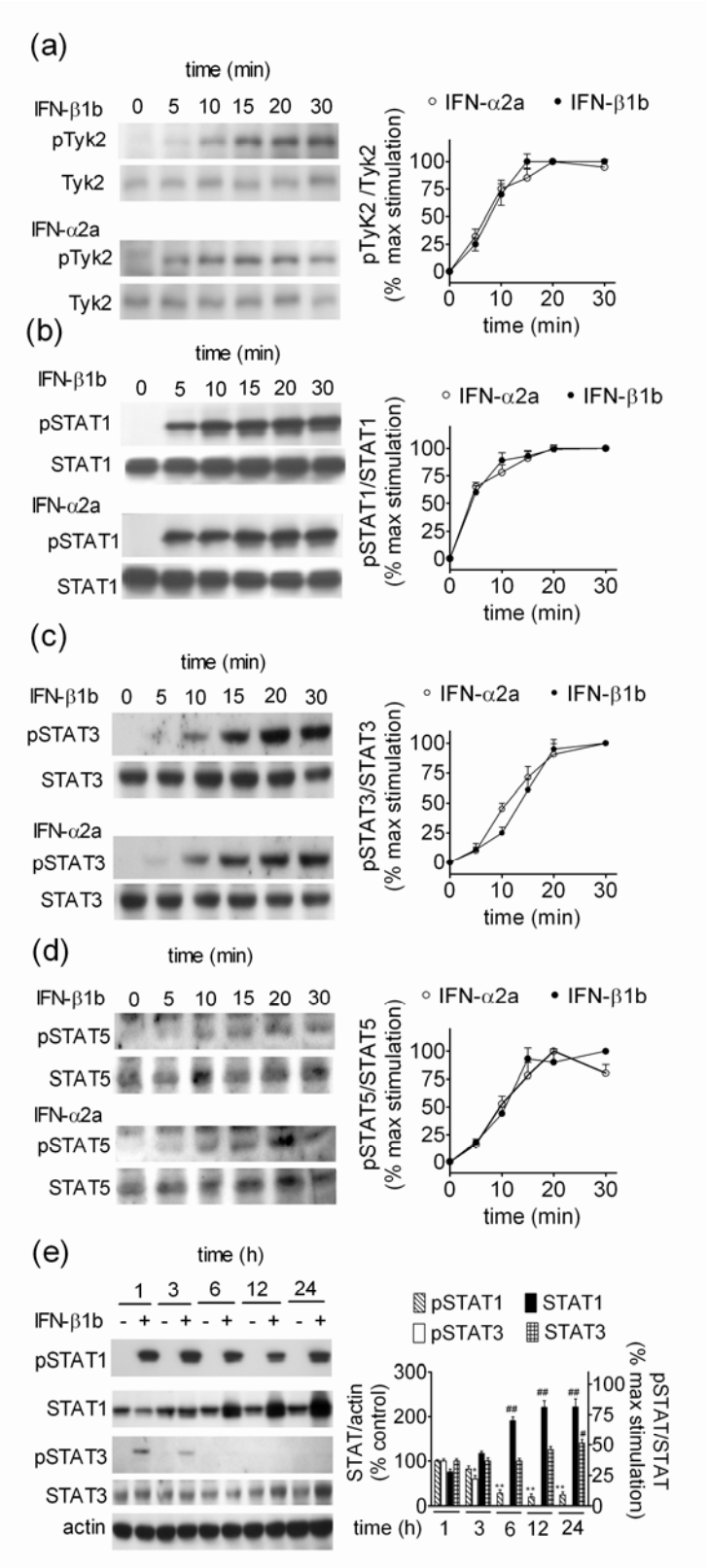


Fig. 12

Type I IFNs induce Tyk2 and STAT phosphorylation in a time-dependent manner. SH-SY5Y cells were treated with either 30 ng/ml of IFN-β1b or IFN-α2a for the indicated times. Zero-time samples were treated with vehicle. The phosphorylated and total forms of Tyk2 (a), STAT1 (b), STAT3 (c) and STAT5 (d) were measured in cell extracts by Western blot. ROD values are expressed as percentage of the maximal stimulation. Data are the mean ± SEM of three-four experiments. (e) Regulation of STAT1 and STAT3 by long-term exposure to IFN-β. Cells were treated with either vehicle or IFN-β1b (30 ng/ml) for the indicated times and the phosphorylated and total forms of STAT1 and STAT3 were measured in cell extracts by Western blot. ROD values of STAT/actin and pSTAT/STAT are reported as percentage of the corresponding control values and as percentage of maximal stimulation, respectively. Data are the mean ± SEM of three experiments. * $p < 0.05$, ** $p < 0.01$ vs the corresponding value at 1 h after IFN; # $p < 0.05$, ## $p < 0.01$ vs control at the same time point. *from: Dedoni et al., 2010*

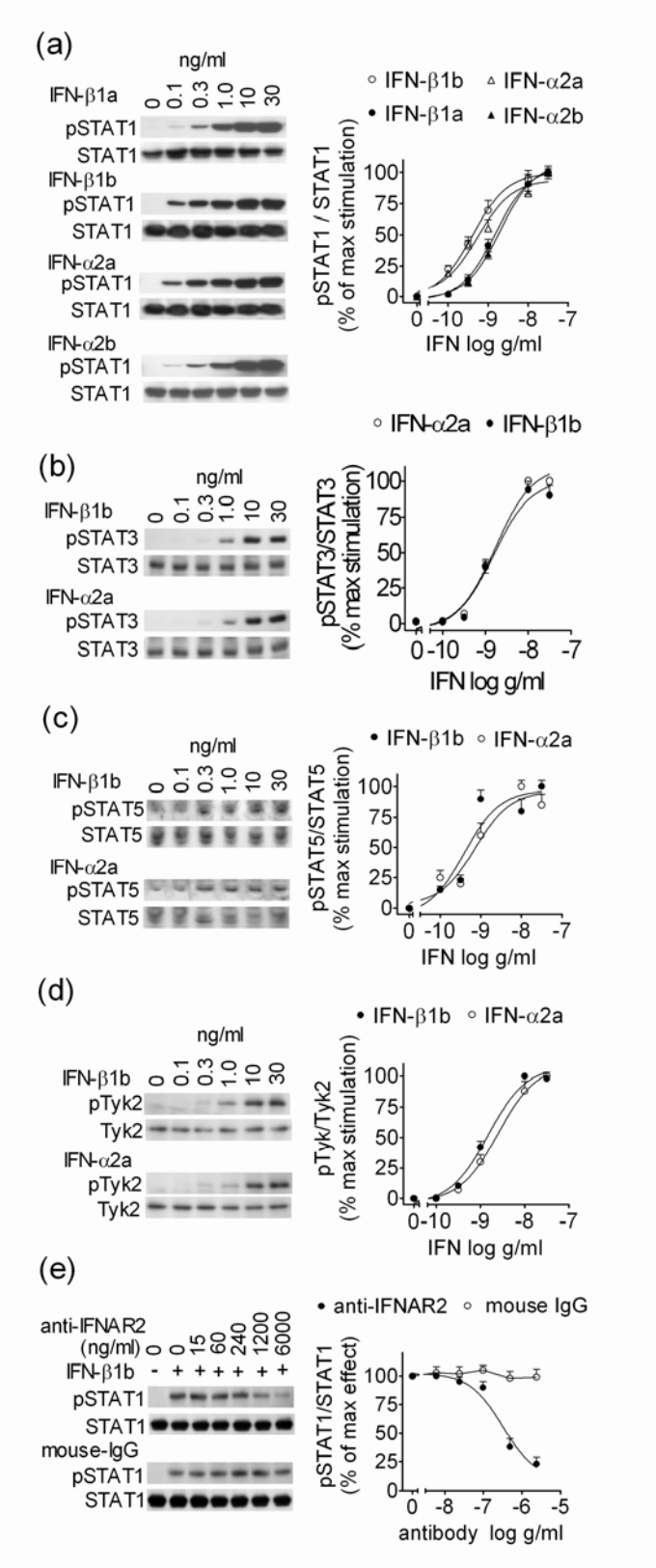


Fig. 13

Concentration-dependent stimulation of Tyk2 and STAT phosphorylation by type I IFNs and blockade by anti-IFNAR2 antibody. Confluent SH-SY5Y cells were treated with either vehicle or the indicated concentrations of each IFN subtype for 15 min. The phosphorylated and total forms of STAT1(a), STAT3 (b), STAT5 (c) and Tyk2 (d) were measured by Western blot and the corresponding ROD values are reported as percentage of maximal stimulation. Data are the mean ± SEM of three experiments. (e) Antagonism of IFN-β-induced STAT1 phosphorylation by anti-IFNAR2 antibody. Cells were pre-treated for 1 h with the indicated concentrations of either anti-IFNAR2 antibody or normal mouse IgG and then incubated with either vehicle or 3 ng/ml IFN-β1b for 15 min. The phosphorylated and total forms of STAT1 were measured and ROD values reported as percentage of the maximal stimulation. Data are the mean ± SEM of three experiments.
from: Dedoni et al., 2010

2.3.3 IFN-β induced neuronal cell death in SH-SY5Y cells

To investigate the effect of type I IFN on human neuronal survival, SH-SY5Y cells were exposed for 24 h to either vehicle or IFN-β (30 ng/ml). Examination of the cells by light microscopy showed that the cytokine treatment altered cell morphology, causing process shortening and cell bodies rounding and shrinkage (Fig. 14a). Fluorescence microscopy analysis of PI and DAPI staining (Fig. 14b,c) indicated that, while vehicle treatment had no effect of cell viability, 24 h exposure to IFN-β induced the appearance of dead cells (control 2.0 ± 0.8, IFN-treated 24 ± 5 % of total nuclei stained by DAPI, $p < 0.01$), and an increase of the number of cells displaying fragmented and condensed nuclei (control 2.5 ± 0.7, IFN-treated 20 ± 3 % of total nuclei, $p < 0.01$), suggesting the occurrence of apoptosis. By using *in situ* TUNEL technique (Fig. 14c), it was observed that in IFN-β-treated samples a higher percentage of cell nuclei showed DNA fragmentation, a hallmark of apoptosis, and this effect was significantly reduced by the addition of the pan-caspase inhibitor Z-VAD-FMK (35 μM), indicating that IFN-induced neuronal death was dependent on caspase activation. The percent values of TUNEL positive nuclei were: control 3.1 ± 0.4, IFN-β 20 ± 1.6 ($p < 0.01$ vs control), Z-VAD-FMK 3.6 ± 0.4, IFN-β + Z-VAD-FMK 12 ± 0.9 ($p < 0.05$ vs IFN-β).

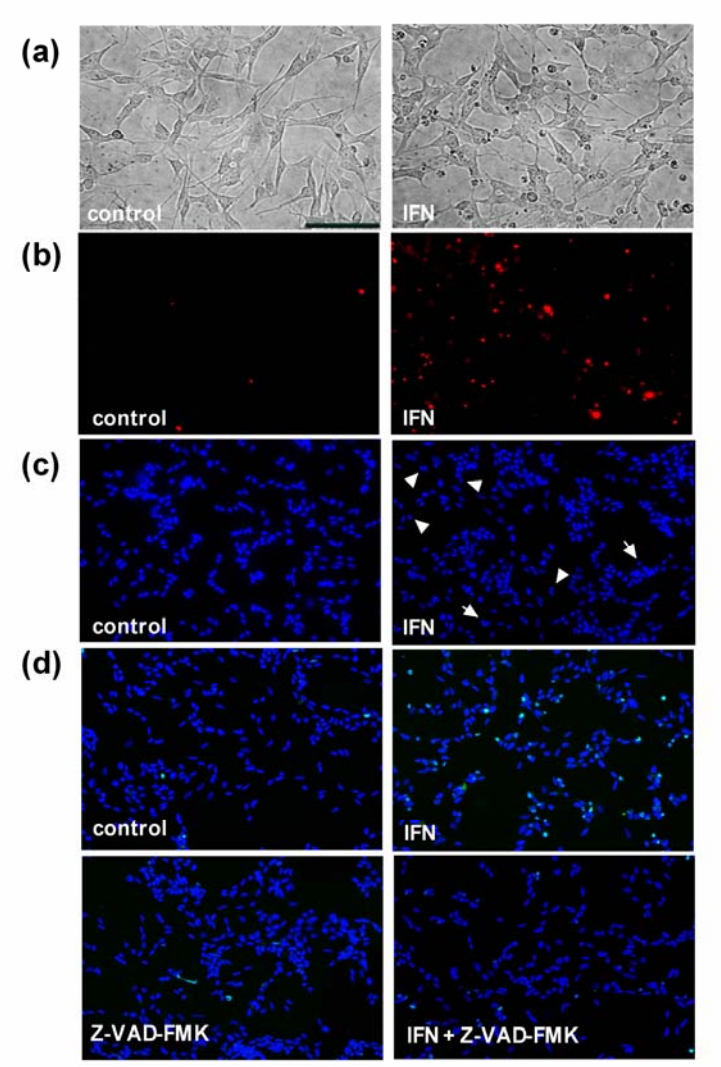


Fig. 14

IFN-β induces apoptosis in SH-SY5Y cells. Cells were incubated for 24 h with either vehicle (control) or IFN-β1b (30 ng/ml) and then examined by light microscopy for morphological analysis (a) and by fluorescence microscopy for PI (b), DAPI (c) and DAPI plus TUNEL labeling (d). In (c) arrows and arrow heads indicate condensed and fragmented nuclei, respectively. In (d) cells were incubated with either vehicle (control), IFN-β, Z-VAD-FMK (35 μM) and IFN-β + Z-VAD-FMK. TUNEL positive nuclei are stained with green or light blue. Micrographs are representative of 3-4 experiments. In (a), scale bar = 100 μm and applies to all micrographs.
from: Dedoni et al., 2010

2.3.4 Effects of IFN on HeLa cell viability.

To explore whether IFNs were also capable of inducing cell death in non-neuronal cells, we examined the sensitivity of HeLa cells to the long-term (24 h) treatment with 30 ng/ml IFN-β, which was observed to cause apoptosis in primary neurons and SH-SY5Y cells. As shown in Fig. 15, the IFN-treatment failed to alter the morphology and viability of HeLa cells.

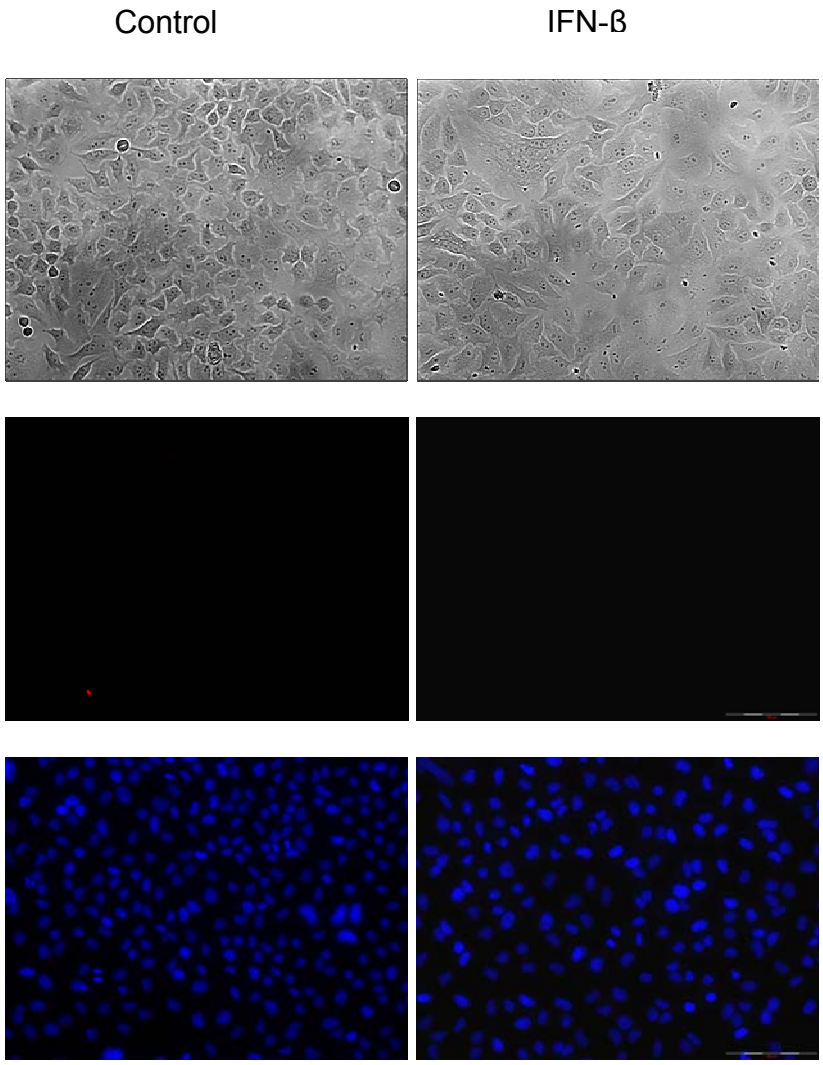


Fig. 15

Long-term treatment with IFN-β fails to affect cell morphology and viability of Hela cells. Cells were incubated for 24 h with either vehicle (control) or IFN-β1b (30 ng/ml) and then examined by light microscopy for morphological analysis (upper panel) and by fluorescence microscopy for PI (red) and DAPI (blue) (lower panels). Scale bar = 100 µm

To assess whether HeLa cells cultured under the described conditions were sensitive to type I IFNs, the ability of the cytokines to induce STAT1 tyrosine phosphorylation was investigated. As reported in Fig. 16, IFN-β1b and IFN-α2b caused a robust increase in phospho-(Tyr701)-STAT1 with EC_{50} values of 109 ± 34 and 133 ± 35 pg/ml (n = 3).

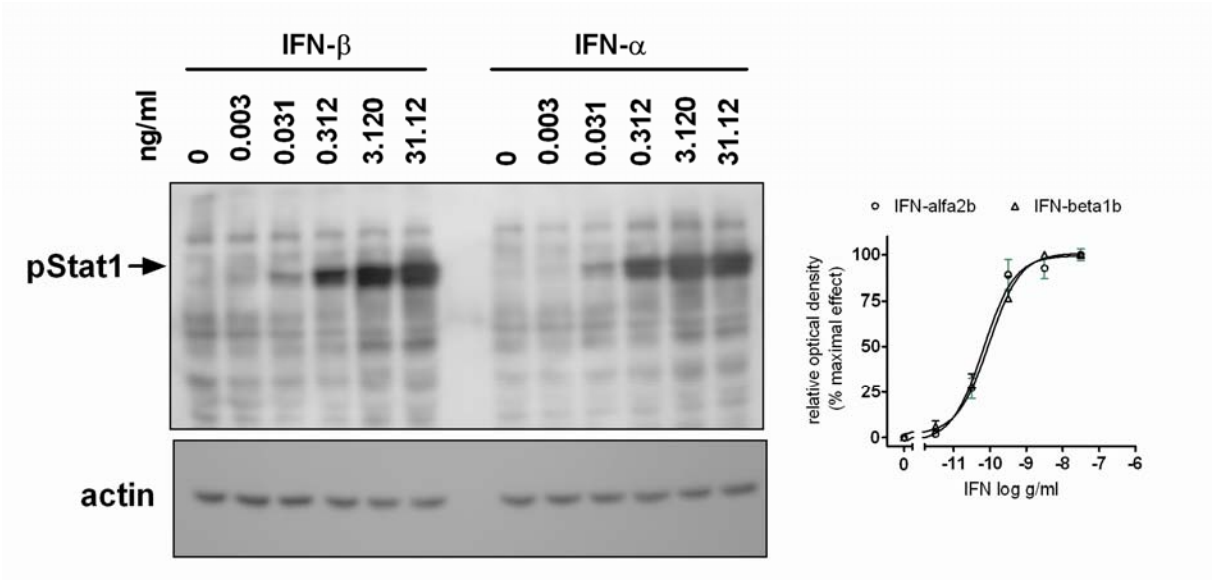


Fig. 16

Concentration-dependent stimulation of STAT1 phosphorylation by type I IFNs in HeLa cells. Confluent cell cultures were treated with either vehicle or the indicated concentrations of each IFN subtype for 15 min. Phospho-(Tyr701)- STAT1 and actin immunoreactivities were measured by Western blot and the corresponding ROD values are reported as percentage of maximal stimulation. Data are the mean ± SEM of three experiments.

2.3.5 IFN-β induced cytochrome C release and caspase activation

In mammalian cells, apoptosis occurs through two major routes, the mitochondrial "intrinsic" apoptotic pathway and the "extrinsic" pathway (Hengartner, 2000). In the "intrinsic" pathway, the loss of outer mitochondrial membrane integrity causes the release of cytochrome C, which binds to and activate the apoptotic protease

activating factor-1, with subsequent activation of caspase-9 and executioner caspases-7, -6 and -3. In the extrinsic pathway, stimulation of cell-surface death receptors initiate apoptosis by activating caspase-8, which then triggers the activation of executioner caspases. To investigate the involvement of these apoptotic pathways in SH-SY5Y cells, the effects of IFN-β on mitochondrial cytochrome C release and caspase activation were examined. In vehicle-treated cells the presence of cytochrome C in the cytosol was almost undetectable, whereas in cells treated with IFN-β there was a marked increase in cytosolic cytochrome C concentration (Fig. 17a). This change was associated with a robust activation of the caspase proteolytic cascade, as indicated by the increase of the cleaved forms of caspase-9 (37 kDa) (Fig. 17b), caspase-7 (20 kDa) (Fig. 17c) and caspase-3 (20 and 17 kDa) (Fig. 17d). Exposure to IFN-β also caused an increased expression of procaspase-7 (Fig. 17c), whereas the levels of procaspase-9 and procaspase-3 were either unaffected or decreased. On the other hand, no detectable expression of either full length caspase-8, its cleaved intermediate p43/p41 or caspase 8 active fragment p18 was observed in cell extracts of control cells and cells treated for 24 h with either 30 ng/ml IFN-β, IFN-α or 25 ng/ml TNF-α (results not shown). These data indicate that IFN-β caused neuronal cell death through the mitochondrial “intrinsic” apoptotic pathway. The activation of the executioner caspases was assessed by examining the cleavage of PARP, a downstream target of activated caspase-3 and caspase-7. IFN-β treatment enhanced the formation of the 89 kDa cleaved form of PARP in a concentration-dependent manner (Fig. 17e). The estimated EC_{50} value of IFN-β (0.86 ± 0.07 ng/ml) correlated with the cytokine potency in stimulating Tyk2 and STAT phosphorylation.

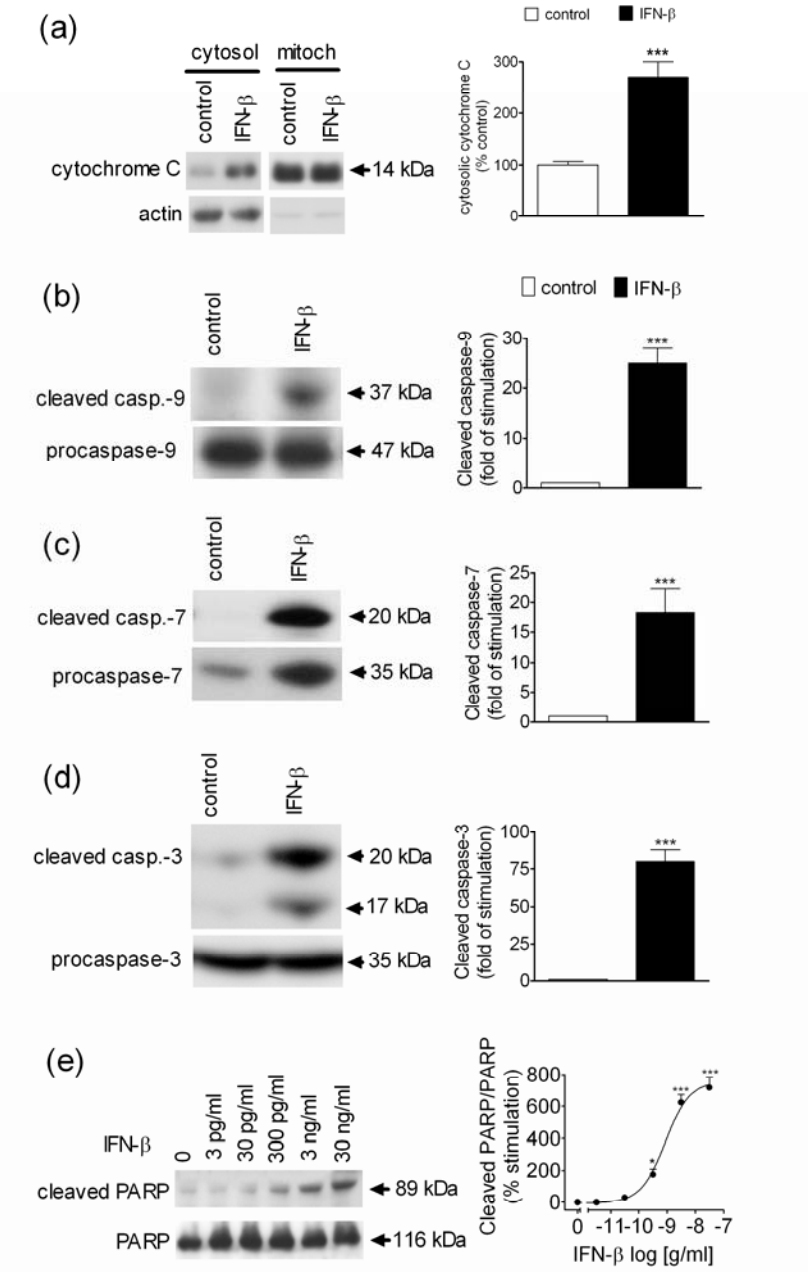


Fig. 17

Induction of cytochrome C release and activation of caspase cascade by IFN-β. SH-SY5Y cells were treated with either vehicle (control) or IFN-β1b (30 ng/ml) for 24 h. (a) Cytochrome C and actin were measured in cytosol and mitochondrial fraction (mitoch) by Western blot. Cytosolic cytochrome C/actin ROD values are reported as percentage of control. Data are the mean ± SEM of three experiments. *** $p < 0.001$. (b-d) Cleaved fragments of caspase-9, -7 and -3 and their corresponding procaspase forms were measured and the ROD values are reported as fold of stimulation. Data are the mean ± SEM of three experiments. *** $p < 0.001$ vs control. (e) Cells were treated with either vehicle or the indicated concentrations of IFN-β1b for 24 h and the cleaved and native forms of PARP were measured by Western blot. ROD values are expressed as percent of stimulation with respect to vehicle-treated control. Data are the mean ± SEM of three experiments. * $p < 0.05$, *** $p < 0.001$.

from: Dedoni et al., 2010

2.3.6 Blockade of JAK activity inhibits IFN-β-induced STAT signaling and caspase activation.

Previous studies in non-neuronal cell types have shown that the JAK-STAT pathway plays a pivotal role in type I IFN regulation of gene expression and cell growth, proliferation and survival (van Boxel-Dezaire *et al*., 2006). The possible involvement of this pathway in IFN-β-induced apoptosis in SH-SY5Y cells was studied by using P6, a pan JAK inhibitor (Thompson *et al*., 2002; Pedranzini *et. al*., 2006). Pre-treatment of the cells for 6 h with P6 (2 μM) significantly attenuated IFN-β stimulation of Tyk2, STAT1 and STAT3 tyrosine phosphorylation (Fig. 18a-c). To assess whether P6-induced inhibition of JAK-STAT signaling translated into impaired STAT-dependent gene transcription, cells were transiently transfected with a luciferase reporter gene containing the ISRE sequence, which is activated by STAT1-STAT2 heterodimers associated with the DNA-binding subunit IRF-9 (de Weerd *et al*., 2007; Schindler *et al*., 2007), In vehicle-treated cells, IFN-β induced a 3-fold increase of ISRE transcriptional activity, which was markedly reduced by pre-treatment with P6 (Fig. 18d). Moreover, cell treatment with P6 significantly reduced the cleavage of caspase-9, -7, -3 and PARP triggered by exposure to IFN-β (Fig. 19a-d).

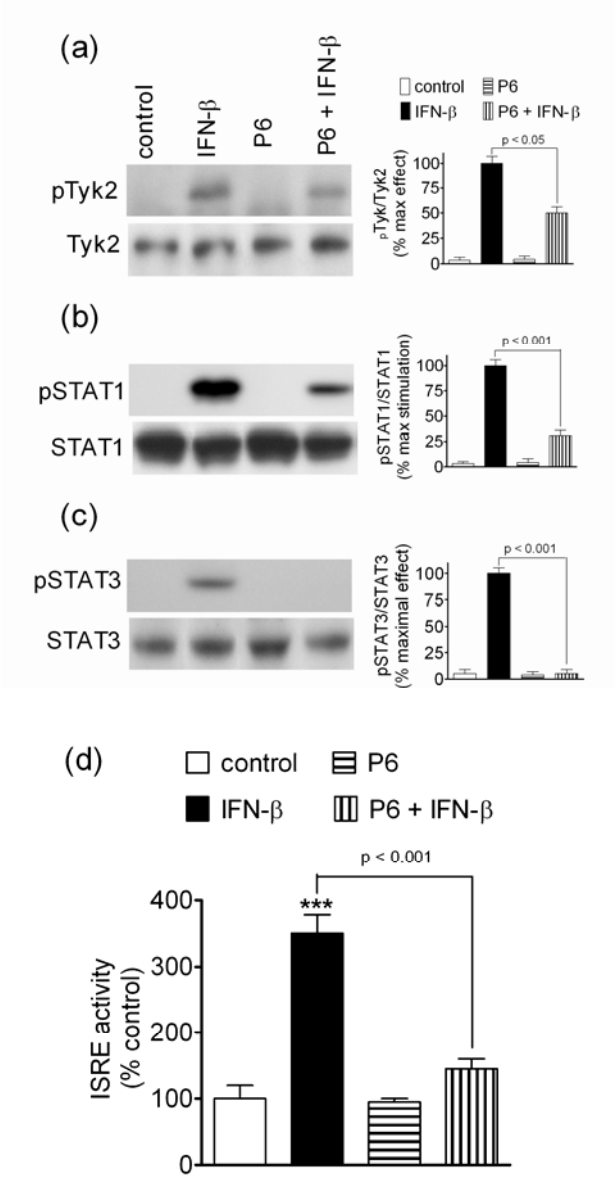


Fig. 18

Blockade of IFN-β-stimulated JAK-STAT signaling by P6. SH-SY5Y cells were treated with either vehicle or P6 (2 μM) for 6 h and then exposed to either vehicle or IFN-β1b (30 ng/ml) for 15 min. (a-c) The phosphorylated and total forms of Tyk2, STAT1 and STAT3 were measured by Western blot and the corresponding ROD values are expressed as percentage of maximal stimulation. Data are the mean ± SEM of three experiments. (d) ISRE transcriptional activity in cells transfected with ISRE-luciferase reporter plasmid, treated with either vehicle or P6 (2 μM) and then exposed to either vehicle or IFN-β1b (30 ng/ml) for 1 h. Data are the mean ± SEM of three separate experiments.
*** $p < 0.001$ vs control.
from: Dedoni et al., 2010

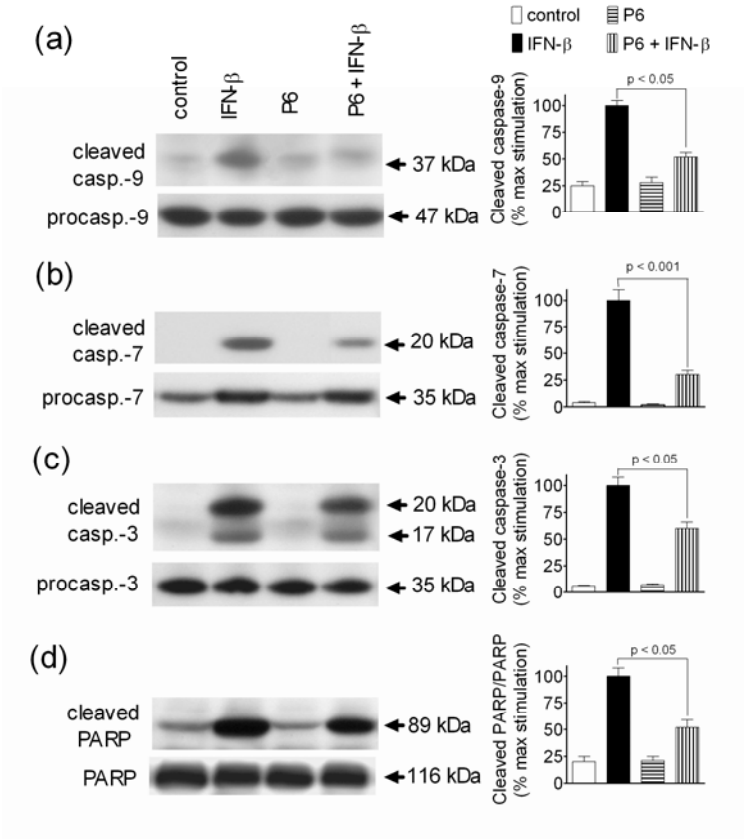


Fig. 19

Blockade of JAK-STAT signaling inhibits IFN-β-stimulated caspase activation. SH-SY5Y cells were treated with either vehicle or P6 (2 μM) for 6 h and then exposed to either vehicle or IFN-β1b (30 ng/ml) for 24 h. (a-d) Cleaved caspase-9, -7, -3 and PARP and the corresponding native forms were measured and the ROD values are reported as percentage of stimulation. Data are the mean ± SEM of three experiments.
from: Dedoni et al., 2010

2.3.7 IFN-β enhances PKR expression and activity

Among the multiple IFN-stimulated genes, PKR is one of the most involved in the regulation of cell growth, differentiation and apoptosis (Samuel *et al*., 1997). PKR mediates translational control in response to dsRNA and other cellular stress stimuli by phosphorylating the α subunit of the translation initiation factor eIF2 on Ser51. Once phosphorylated by PKR, eIF-2α enhances the affinity for GDP and thus inhibits translation initiation events. Prolonged IFN-β treatment of SH-SY5Y cells increased the expression of PKR, which was detected at 6 h and persisted for at least 24 h (Fig. 20a). IFN-β-stimulated PKR induction was completely prevented by pre-treatment with either P6 (2 μM) (Fig. 20b) or the transcription inhibitor actinomycin D (100 ng/ml for 3 h) (results not shown). Moreover, cell transfection with STAT1 siRNA reduced

basal and IFN-β-stimulated STAT1 expression by 60 and 50%, respectively, and completely blocked the increase in PKR levels elicited by IFN-β (Fig. 21). IFN-β treatment also enhanced the phosphorylation of eIF-2α, indicating an increased PKR activity (Fig. 20c). To investigate the role of PKR in neuronal apoptosis elicited by IFN-β, a chemical inhibitor of PKR (Jammi *et al*., 2003; Shimazawa and Hara, 2006) was employed. Pre-treatment of SH-SY5Y cells with PKR inhibitor (1 μM) significantly reduced IFN-β-stimulated eIF-2α phosphorylation (Fig. 20c) and curtailed IFN-β-induced activation of caspase-7 and PARP proteolysis (Fig. 20d,e).

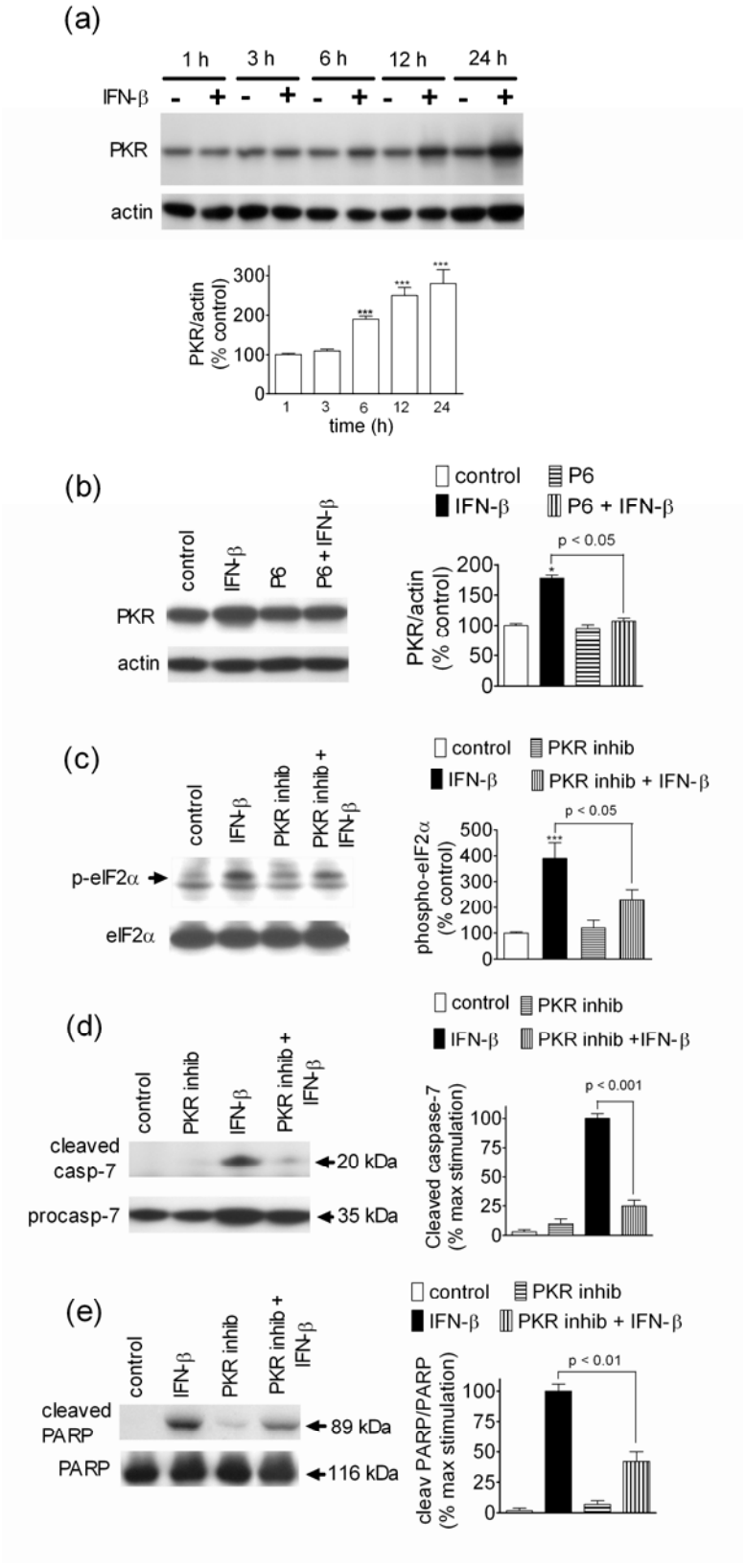


Fig. 20

PKR is induced by IFN-β and promotes caspase activation. (a) Time-dependent enhancement of PKR expression by IFN-β. SH-SY5Y cells were treated for the indicated times with either vehicle or IFN-β1b (30 ng/ml) and PRK and actin immunoreactivities were analyzed by Western blot. ROD values of IFN-treated samples are reported as percentage of control values at each time point. Data are the mean ± SEM of three experiments; *** $p < 0.001$ vs the corresponding control value. (b) Blockade of PKR induction by P6. Cells were treated with either vehicle or P6 (2 μM) for 3 h and then exposed to either vehicle or IFN-β1b (30 ng/ml) for 6 h. ROD values are reported as percent of control and are the mean ± SEM of three experiments; * $p < 0.05$ vs control. (c) IFN-β enhances PKR activity. Cells were incubated for 1 h with either vehicle (control) or 1 μM PKR inhibitor (PKR inhib) and then with either vehicle or IFN-β1b (30 ng/ml) for 24 h. Cell extracts were analyzed by Western blot with antibodies to phospho-Ser51-eIF2α and total eIF2α. ROD values are reported as percentage of control. Data are the mean ± SEM of three experiments. *** $p < 0.001$ vs control. (d,e) Inhibition of PKR prevents IFN-β-induced caspase-7 and PARP cleavage. Cells were incubated for 1 h with either vehicle (control) or 1 μM PKR inhibitor and then with either vehicle or IFN-β1b (30 ng/ml) for 24 h. Cleaved caspase 7, procaspase-7, cleaved PARP and PARP were analyzed by Western blot. ROD values are reported as percentage of maximal effect. Data are the mean ± SEM of three experiments.

from: Dedoni et al., 2010

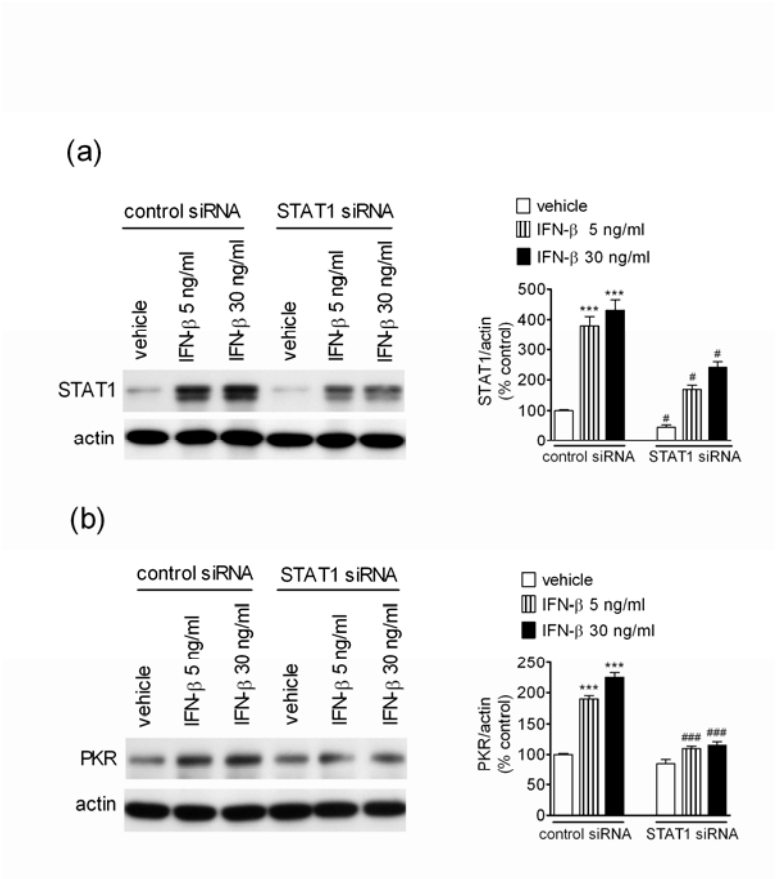


Fig. 21

Knockdown of STAT1 prevents PKR induction by IFN-β. Cells were transfected with either control siRNA or STAT1 specific siRNA and 48 h later incubated for 6 h with either vehicle or the indicated concentrations of IFN-β. Cell extracts were then analyzed for STAT1 (a) and PKR (b) expression by Western blot. The results are the mean ± SEM of three experiments. *** $p < 0.001$ vs control (control siRNA + vehicle); # $p < 0.05$; ### $p < 0.001$ vs the corresponding value in control siRNA treated samples.
from: Dedoni et al., 2010

2.3.8 Involvement of p38 MAPK in type I IFN-induced apoptosis

Mitogen-activated protein kinases (MAPK) are a group of widely expressed serine-threonine protein kinases that mediate cellular responses to different extracellular signals (Schaeffer and Weber, 1999). Three distinct families of MAPK have been identified: the extracellular-signal regulated kinases (ERK), the Jun N-terminal kinases (JNK) and the p38 MAPK. There are four p38 isoforms with high sequence homology and are designated p38α, p38β, p38γ and p38δ (Schaeffer and Weber, 1999). It has been shown that, like JNK, p38 MAPK are activated by different stressful stimuli, including UV irradiation, heat, osmotic shock, genotoxic agents, toxins, and cytokines (Kumar *et al*., 2003; Wagner e Nebreda, 2009). Previous

studies have shown that in non-neuronal cells type I IFN can induce p38 MAPK activation through a signaling cascade possibly involving JAK, the guanine exchange factor Vav, the small G protein Rac1, a MAPK kinase kinase and the MAPK kinases (MEK) 3 and 6 (Platanias, 2003). However, whether type I IFN can regulate the p38 MAPK system in neuronal cells has not yet been investigated. As shown in Fig. 22, exposure of SH-SY5Y cells to IFN-β (30 ng/ml) caused a time-dependent increase of dual p38 MAPK phosphorylation on Thr180/Tyr182, which is associated with an enhanced kinase activity. The IFN stimulation became significant at 3 h and persisted for at least 24 h.

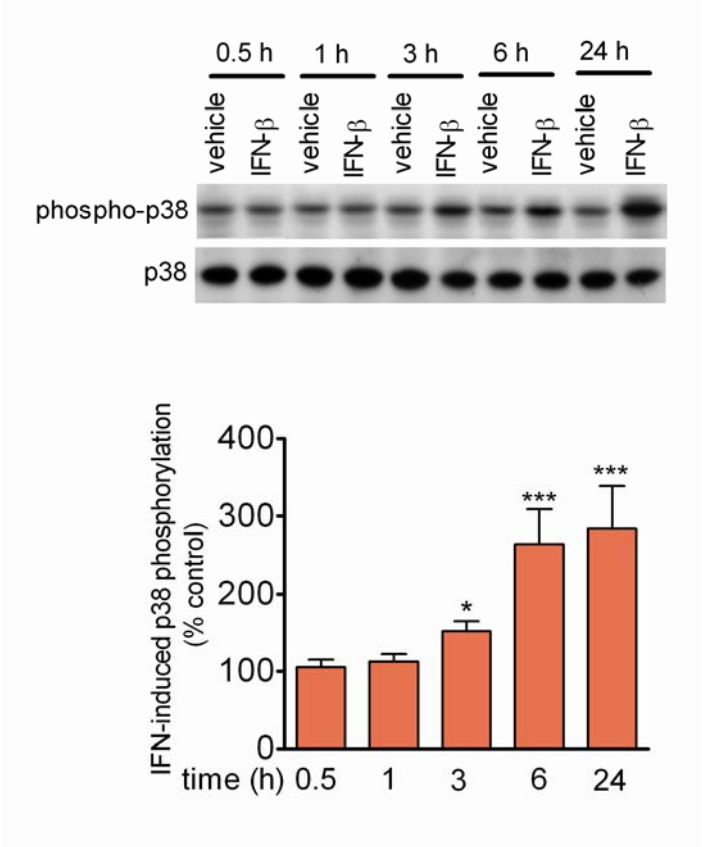


Fig. 22

IFN-β induces a long-lasting increase in p38 MAPK phosphorylation. SH-SY5Y cells were incubated in a medium containing 1 % FCS in the presence of either vehicle or 30 ng/ml IFN-β1b for the indicated periods of time. Values are the mean ± SEM of three experiments. * $p < 0.05$, *** $p < 0.001$ vs the respective control

To investigate whether IFN regulation of p38 MAPK involved JAK activity, cell were pre-treated with the pan-JAK inhibitor P6. Exposure to 2 μM P6 for 6 h significantly reduced the induction of p38 MAPK phosphorylation induced by IFN-β (Fig. 23).

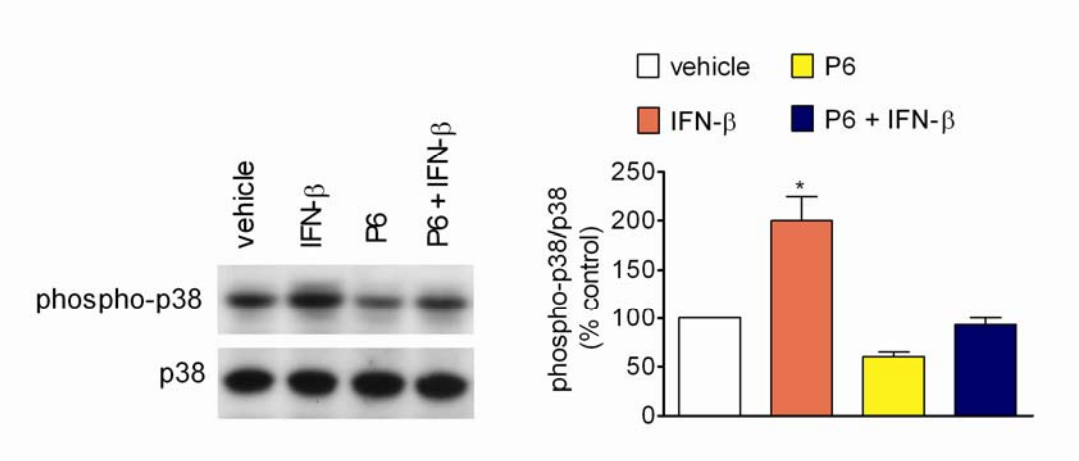


Fig. 23

The pan-Janus kinase (JAK) inhibitor pyridone 6 (P6) attenuates IFN-β-induced p38 MAPK phosphorylation. SH-SY5Y cells were treated with either vehicle or 2 μM P6 for 6 h and then exposed for 24 h to either vehicle or 30 ng/ml IFN-β. * $p < 0.05$ vs control.

These data indicated that in SH-SY5Y cells type I IFN could regulate p38 MAPK activity in a JAK-dependent manner. To gain more information on the role of this protein kinase in IFN action, the effects of a specific p38 MAPK inhibitor SB 203580 on IFN-induced apoptosis of SH-SY5Y cells were examined. As shown in Figs. 24-26, cell treatment with 10 μM SB 203580 caused a significant inhibition of IFN-β-induced caspase-7 cleavage, PARP proteolysis and DNA fragmentation.

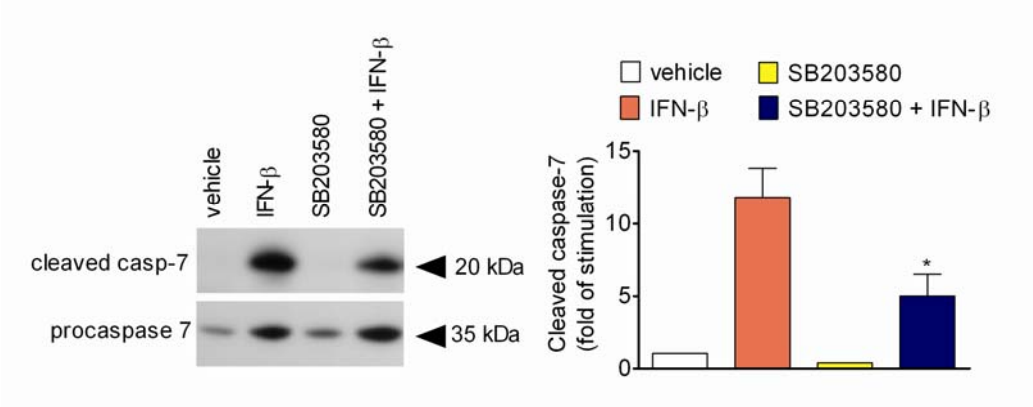


Fig. 24

The p38 MAPK inhibitor SB203580 attenuates IFN-β-induced caspase-7 activation. SH-SY5Y cells were treated with either vehicle or 10 μM SB203580 for 1 h and then exposed to either vehicle or IFN-β (30 ng/ml) for 24 h. Values are the mean ± SEM of three experiments. * $p < 0.05$ vs IFN-β alone.

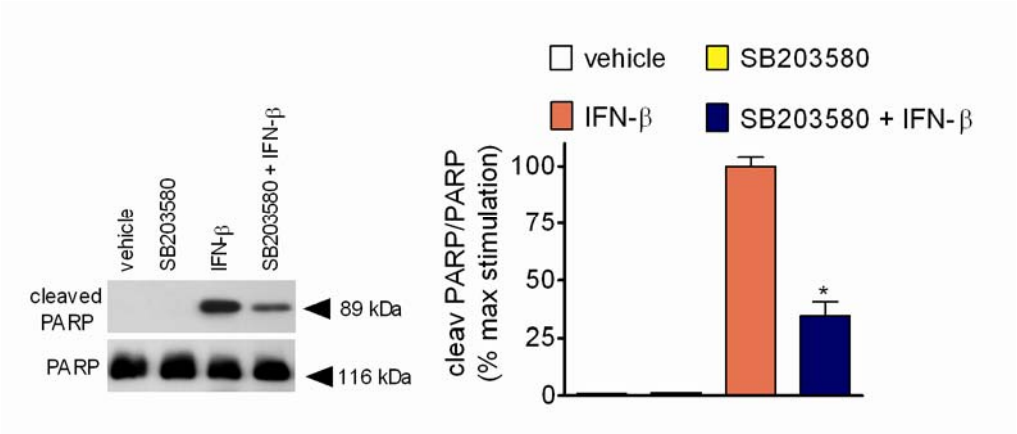
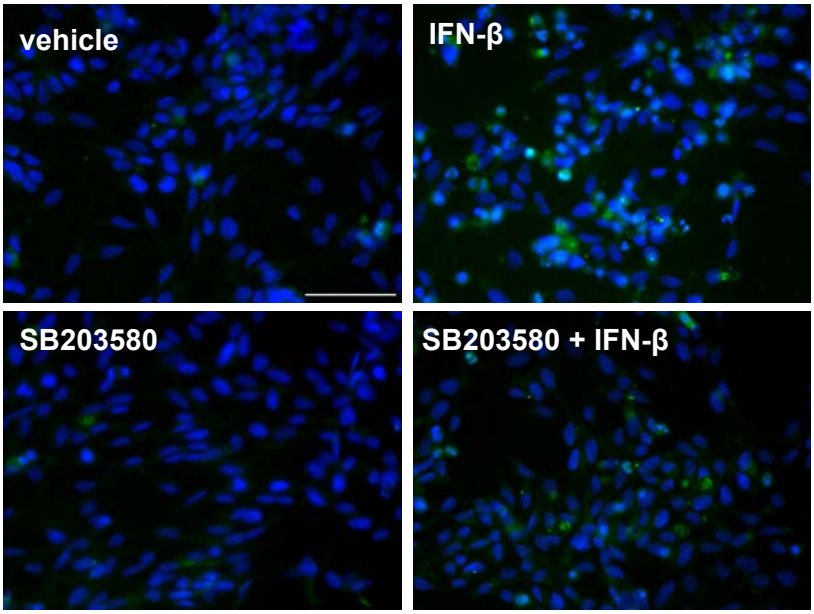


Fig. 25

SB203580 attenuates IFN-β-induced poly-(ADP ribose) polymerase (PARP) cleavage. SH-SY5Y cells were treated with either vehicle or 10 μM SB203580 for 1 h and then exposed to either vehicle or IFN-β (30 ng/ml) for 24 h. Values are the mean ± SEM of three experiments. * $p < 0.05$ vs IFN-β alone.



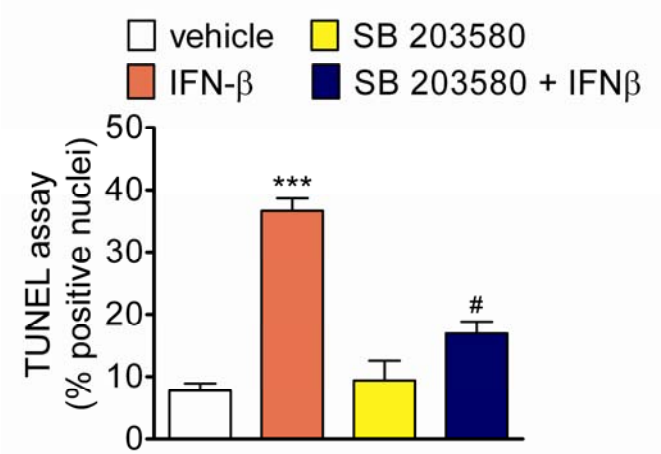


Fig. 26

Inhibition of p38 MAPK reduces IFN-β-induced DNA fragmentation. SH-SY5Y cells grown on glass coverslips were incubated with either vehicle or 10 μM SB203580 for 1 h and then treated with either vehicle or 30 ng/ml IFN-β for 24 h. The percentage of apoptotic cell nuclei was determined by in situ terminal transferase dUTP nick end-labeling (TUNEL) assay. Values are the mean ± SEM of three experiments. *** $p < 0.001$ vs control, # $p < 0.001$ vs IFN-β alone

2.3.9 Long-term treatment with IFN-β induces down-regulation of PI3K/Akt pathway

In addition to the conventional JAK-STAT signaling, short-term treatment with type I IFNs has been found to activate the PI3K/Akt pathway, an event associated with pro-survival effects in different cell types (van Boxel-Dezaire *et al*., 2006). In agreement with these studies, exposure of SH-SY5Y cells to IFN-β for 10 min caused a significant increase in Thr308 and Ser473 phosphorylation of Akt, a main downstream effector of PI3K, indicating an enhanced PI3K activity (Fig. 27a). However, 24 h treatment with IFN-β produced a decrease of Akt phosphorylation (Fig. 27a), which was associated with decreased Akt activity, as indicated by the reduced phosphorylation of exogenously added GSK-3α/β fusion protein substrate (Fig. 27b). Moreover, long-term treatment with IFN-β inhibited the Ser9 phosphorylation of endogenously expressed GSK-3β (Fig. 27c). Similarly, treatment of primary neurons with IFN-β for 24 h caused a significant decrease in the levels of phospho-(Thr308)-Akt and phospho-(Ser9)-GSK-3β (Fig. 28).

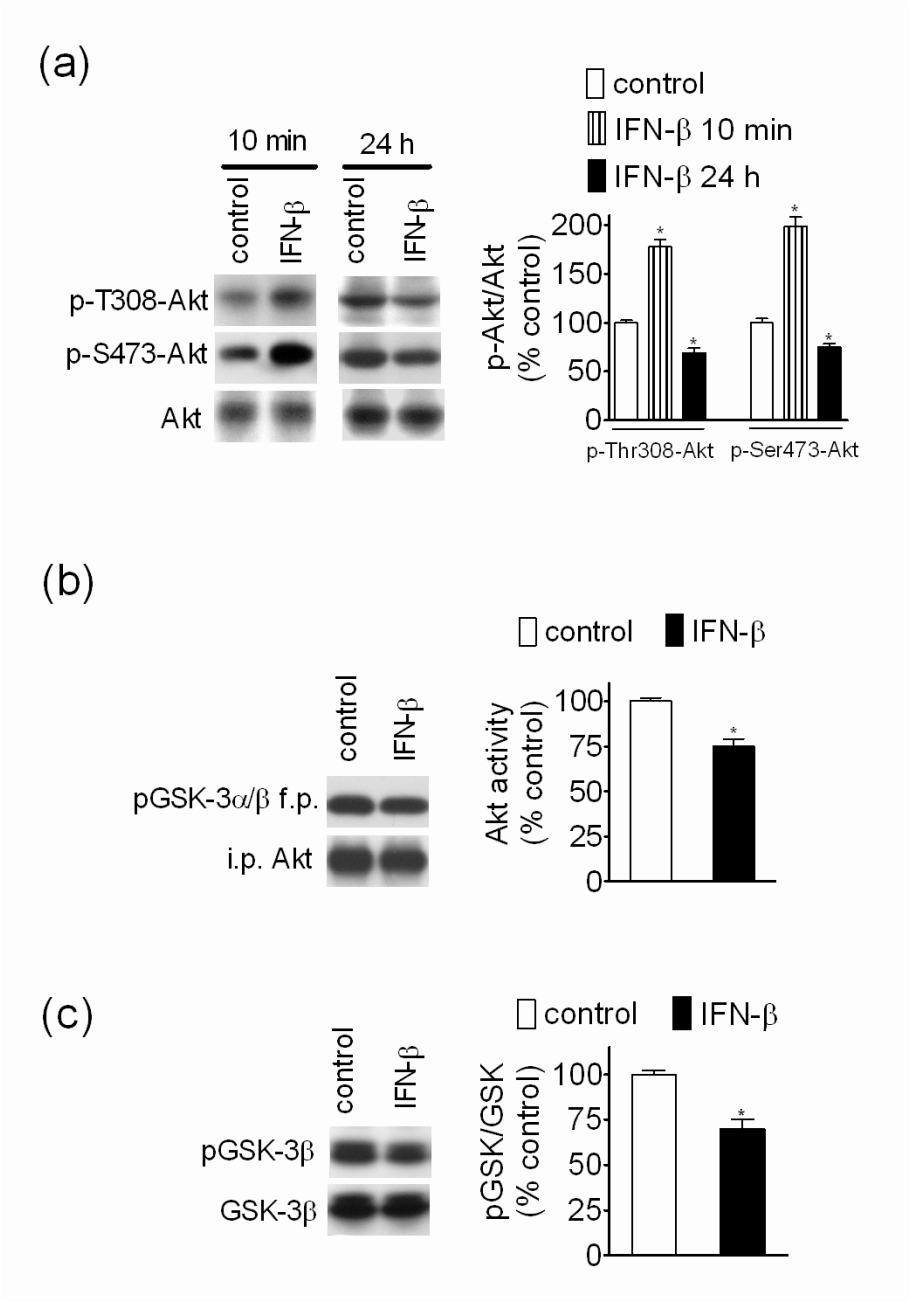


Fig. 27

Long-term IFN-β exposure down-regulates PI3K/Akt signaling in SH-SY5Y cells. (a) Opposite effect on Akt phosphorylation following short-term (10 min) and long-term (24 h) exposure to IFN-β (30 ng/ml). Phospho-Thr308-Akt (pT308-Akt), phospho-Ser473-Akt (pS473-Akt) and total Akt were analyzed by Western blot and ROD values were expressed as percentage of the respective control. Data are the mean ± SEM of three experiments; * $p < 0.05$ vs the corresponding control value. (b) Down-regulation of Akt activity following long-term IFN-β exposure. Akt was immunoprecipitated from cell extracts of vehicle (control) and IFN-β-treated (30 ng/ml for 24 h) samples and the kinase activity was assayed by measuring the phosphorylation of the substrate GSK-3α/β fusion protein (pGSK-3α/β f.p.) by Western blot. The optical density of each band was normalized to the corresponding density of immunoprecipitated Akt. Data are the mean ± SEM of four experiments. * $p < 0.05$ vs control. (c) Reduced endogenous GSK-3β (pGSK-3β) Ser9 phosphorylation by IFN-β treatment. Cells were incubated for 24 h with either vehicle (control) or IFN-β1b (30 ng/ml) and pGSK-3β and total GSK-3β were measured by Western blot. Data are the mean ± SEM of three experiments. * $p < 0.05$ vs control.
Modified from: Dedoni et al., 2010

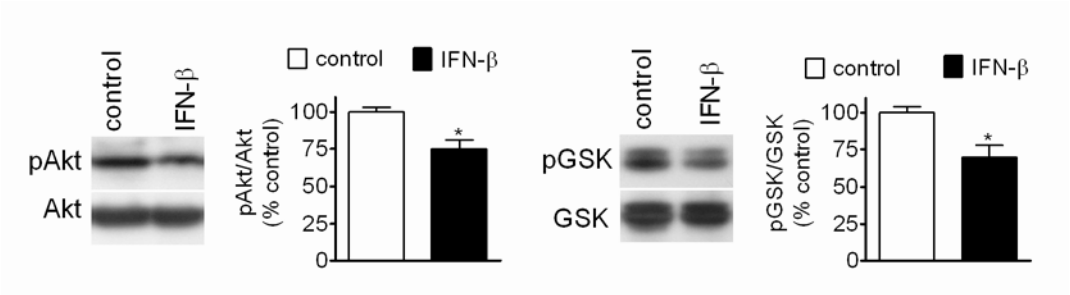


Fig. 28

Reduction of Akt and GSK-3β phosphorylation by long-term exposure of mouse primary cortical neurons to IFN-β. Cell cultures were treated for 24 h with either vehicle (control) or mouse IFN-β (30 ng/ml) and phospho-(Thr308)-Akt, phospho-(Ser9)-GSK-3β and the corresponding total forms were measured by Western blot. ROD values are expressed as percent of control and are the mean ± SEM of three experiments. * $p < 0.05$ vs control.
Modified from: Dedoni et al., 2010

SH-SY5Y cell exposure to IGF-1 (10 ng/ml), which activates receptors coupled to PI3K, caused an acute increase of phospho-(Thr308)-Akt and phospho-(Ser9)-GSK-3β levels, which was significantly attenuated in cells pre-treated with IFN-β for 24 h (Fig. 29). As tyrosine phosphorylation of the adaptor protein IRS-1 is involved in both type I IFN and IGF-1 signaling to PI3K (Myers *et al.,* 1993), it was important to examine whether the long-term exposure to IFN-β affected IRS-1 phosphorylation. Immunoprecipitation experiments indicated that prolonged treatment with IFN-β significantly reduced the basal level of phospho-Tyr-IRS-1 and the stimulation induced by IGF-1 (Fig. 30). To investigate the relevance of down-regulation of PI3K-Akt signaling in IFN-β-induced apoptosis, SH-SY5Y cells were transiently transfected with myr-Akt1, a constitutively active construct of Akt (Kohn *et al*., 1996) and analyzed with *in situ* TUNEL assay. As shown in (Fig. 31), IFN-β treatment increased the percent of apoptotic cells in vector-transfected samples and this response was significantly reduced in cells expressing the constitutively active form of Akt1.

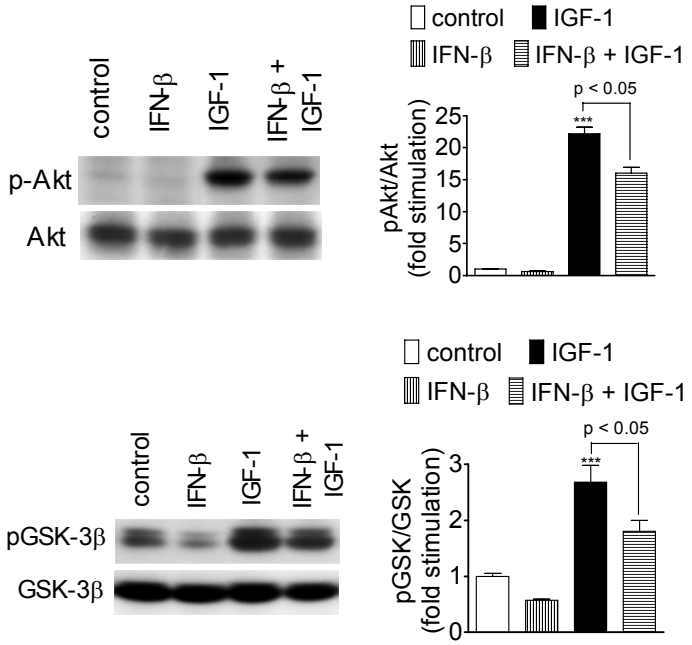


Fig. 29

Inhibition of IGF-1-induced PI3K/Akt signaling by long-term IFN-β treatment. Cells were incubated for 24 h with either vehicle (control) or IFN-β1b (30 ng/ml) and then exposed for 10 min to either vehicle or IGF-1 (10 ng/ml). Phospho-Akt (pAkt), pGSK-3β, Akt and GSK-3β were measured by Western blot and ROD values expressed as percentage of control (vehicle + vehicle). Data are the mean ± SEM of three experiments. *** $p < 0.001$ vs control.
Modified from: Dedoni et al., 2010

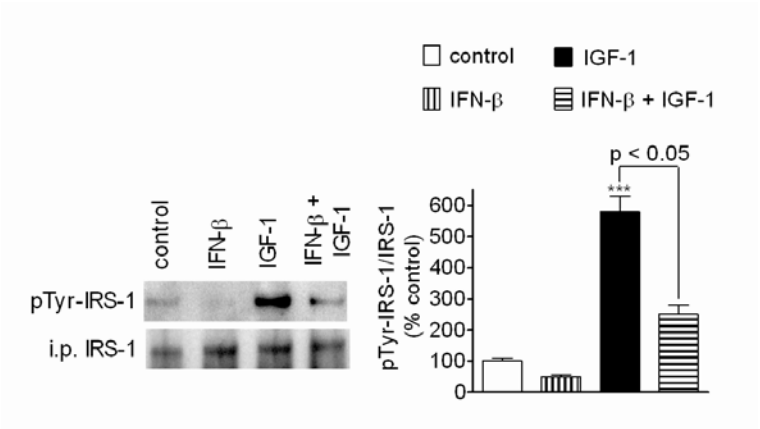


Fig. 30

Long-term IFN-β treatment attenuates IRS-1 tyrosine phosphorylation. Cells were incubated for 24 h with either vehicle (control) or IFN-β1b (30 ng/ml) and then exposed for 10 min to either vehicle or IGF-1 (10 ng/ml). IRS-1 was immunoprecipitated from cell extracts and the level of IRS-1 tyrosine phosphorylation was determined by Western blot. The optical density of each band was normalized to the corresponding density of immunoprecipitated IRS-1 (i.p. IRS-1). Data are the mean ± SEM of three experiments. *** $p < 0.001$ vs control.
Modified from: Dedoni et al., 2010

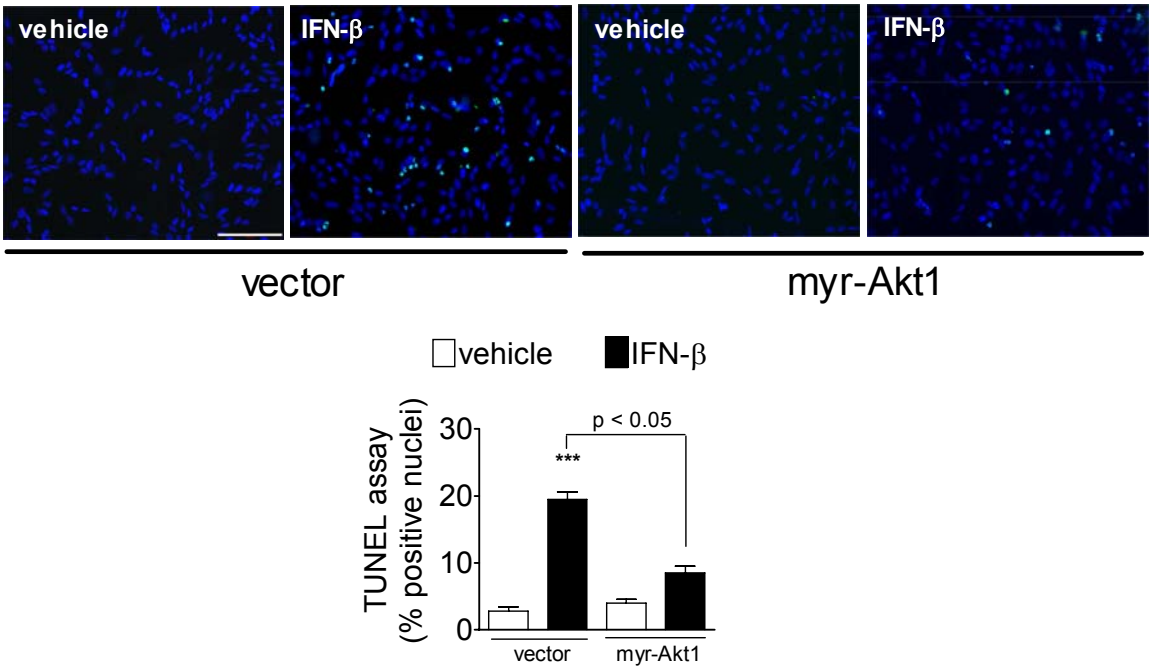


Fig. 31

Constitutively active Akt1 attenuates IFN-β-induced apoptosis. Cells were transfected with either empty vector or a constitutively active construct of Akt1 (myr-Akt1) and then treated with vehicle or IFN-β1b (30 ng/ml) for 24 h. The percentage of apoptotic cell nuclei was determined by *in situ* TUNEL assay. Data are the mean ± SEM of three experiments. *** $p < 0.001$ vs empty vector + vehicle. Scale bar = 100 μm.
Modified from: Dedoni et al., 2010

2.3.10 GSK-3 inhibition prevents PKR induction and apoptosis by IFN-β

Recent studies have demonstrated that GSK-3β is not only involved in glucose metabolism but also regulates the apoptosis cascade in several cell types (Beurel *et al*, 2006). This kinase is constitutively active and its activity is inhibited by phosphorylation on Ser9. The finding that long-term exposure to IFN caused an evident decrease in GSK-3β phosphorylation on Ser9, indicated an up-regulation of GSK-3β activity. This observation raised the question as to whether this protein kinase was an essential component of the IFN-triggered programmed cell death.

To address this issue, the compounds SB216763 and AR A014418, two structurally different inhibitors of GSK-3α and GSK-3β, were used. Pre-treatment of SH-SY5Y cells with SB216763 almost completely prevented caspase-9, - 7 and -3 activation caused by 24 h exposure to IFN-β (Fig. 32).

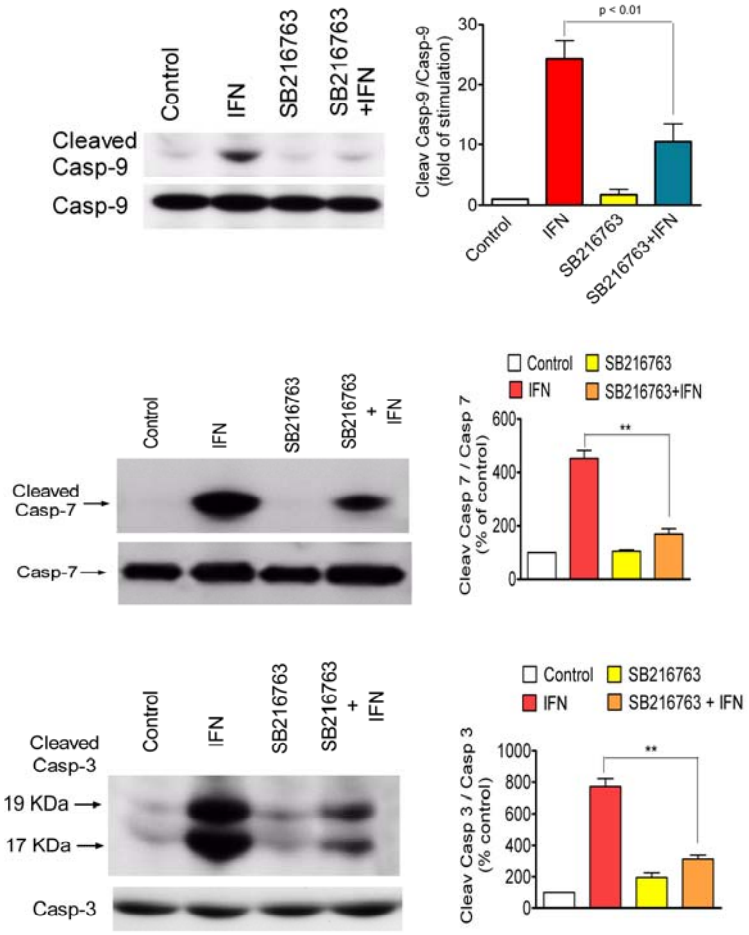


Fig. 32

The GSK-3 inhibitor SB216763 prevent caspases activation by IFN-β.Cells were preincubated for 1 h with either vehicle (control) or 5 μM SB 216763 and then treated with either vehicle or IFN-β1b (30 ng/ml) for 24 h. Cell extracts were prepared and analyzed for caspase cleavage by Western blot. Values are the mean ± SEM of three experiments.

Similarly, cell treatment with AR A014418 attenuated IFN-β-induced increase of PKR expression and activation of caspase-7 (Fig. 33).

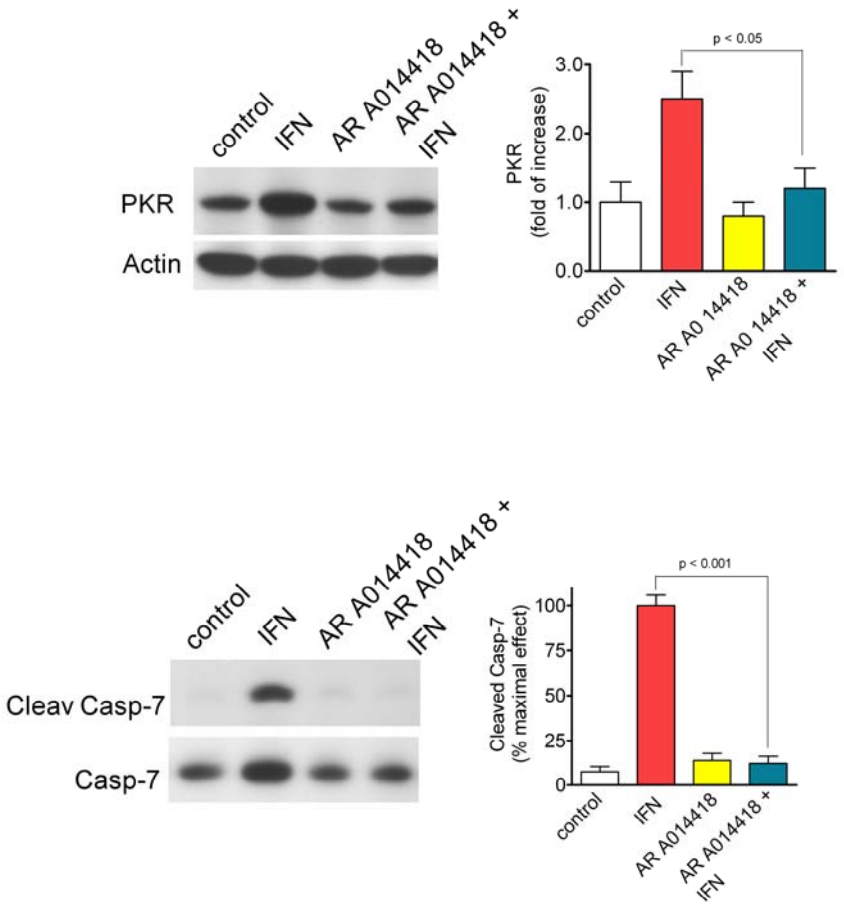


Fig 33

The GSK-3 inhibitor AR A014418 prevents PKR induction and caspase-7 activation by IFN-β. Cells were preincubated for 1 h with either vehicle (control) or 10 μM AR 0A14418 and then treated with either vehicle or IFN-β1b (30 ng/ml) for 24 h. Cell extracts were prepared and analyzed for PKR expression and caspase-7 cleavage by Western blot. Values are the mean ± SEM of three experiments.

In addition, analysis of cell DNA fragmentation by the TUNEL assay indicated that cell pre-treatment with SB216763 significantly reduced the percentage of cells undergoing apoptosis following treatment for 24 h with IFN-β (Fig. 34).

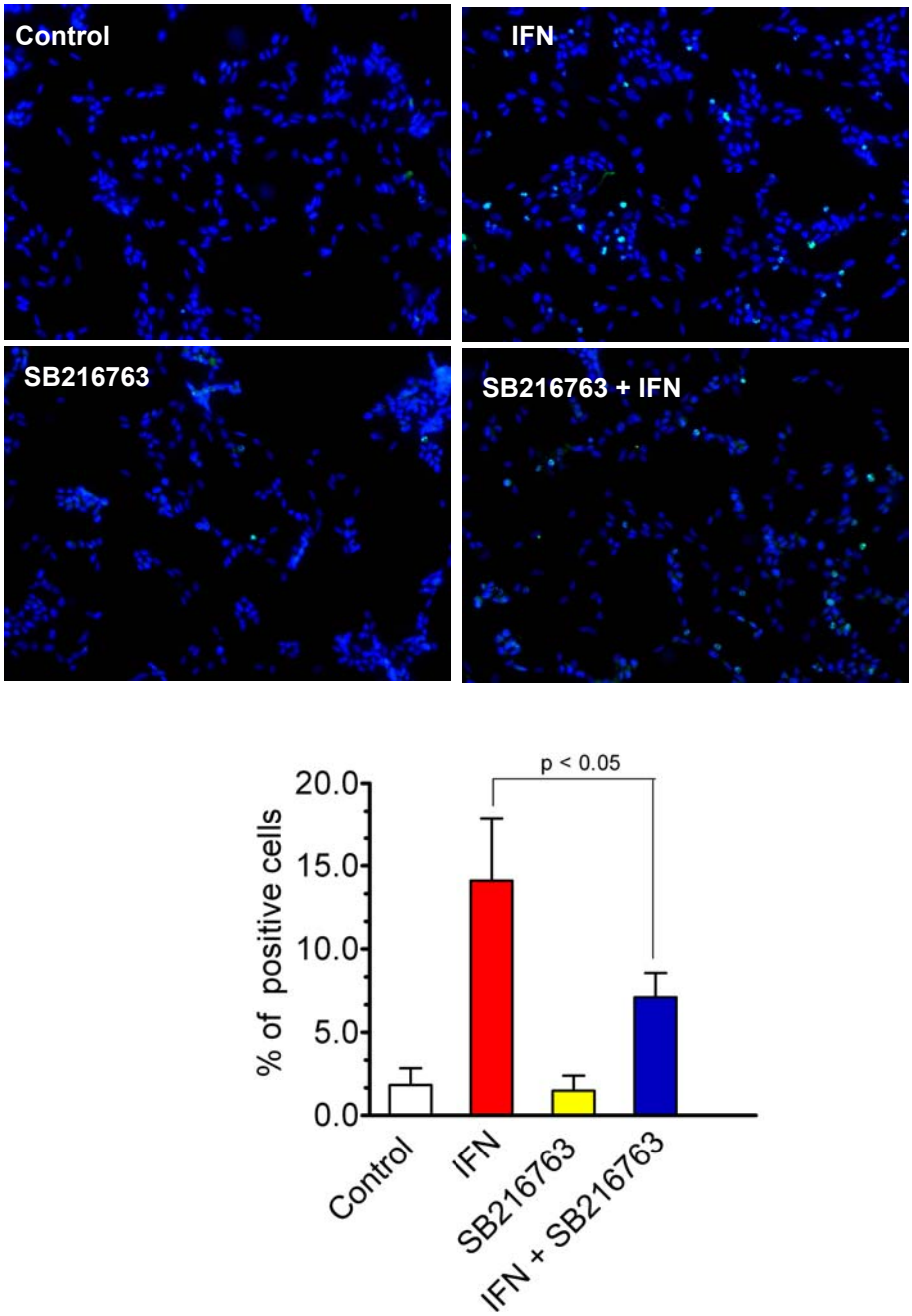


Fig. 34

The GSK-3 inhibitor SB 216763 reduces IFN-induced DNA fragmentation. Cells were preincubated for 1 h with either vehicle (control) or 5 μM SB 216763 and then treated with either vehicle or 30 ng/ml IFN-β1b for 24 h. Cells were then washed, fixed and incubated with fluorescein-12-dUTP and recombinant Terminal Deoxynucleotidyl Transferase. Cell nuclei were stained with DAPI. Cells were analyzed by fluorescence microscopy. The cell nuclei showing a green fluorescence were counted and their number was reported as percent of total nuclei. Values are the mean ± SEM of three determinations.

2.3.11 Long-term type I IFN treatment inhibits BDNF receptor signaling and neuritogenesis.

A number of studies have linked a dysregulation of brain-derived neurotrophic factor (BDNF) receptor signaling to the pathogenesis of different neurodegenerative diseases and neuropsychiatric disorders (Hashimoto *et al*., 2004; Hu and Russek, 2008). It has been reported that BDNF action could be impaired in depression, stress-related affective disorders and schizophrenia. In postmortem brain samples of depressed and schizophrenic patients BDNF levels have been found to be reduced (Weickert *et al.,* 2003; Hashimoto *et al.,* 2005). Moreover, in humans chronic administration of several antidepressants, including selective serotonin reuptake inhibitors, increases and restores BDNF expression (Nibuya *et al*., 1995; Chen *et al*., 2001; Castrèn *et al*., 2004; Kozisek *et al.,* 2008).

BDNF exerts its biological actions through the stimulation of a specific cell-surface receptor, which belongs to the family of the tyrosine kinase-coupled receptors, known as tropomyosin receptor kinase B (TrkB).

Previous studies have shown that SH-SY5Y cells differentiated with RA express TrkB receptors and are responsive to the neurotrophic effects of BDNF (Kaplan *et al*., 1993; Encinas *et al*., 1999; Xie *et al*., 2010). Therefore, it was of interest to investigate whether long-term exposure of differentiated SH-SH5Y to type I IFNs could affect BDNF-induced responses, such as TrkB signaling and neuronal differentiation.

As shown in Fig. 35, short-term exposure of RA-differentiated SH-SY5Y cells to BDNF (0.9 nM) elicited a marked increase of Akt phosphorylation at Thr308, which was associated with an increase in the inhibitory phosphorylation of GSK-3β at Ser9.

Both BDNF responses were significantly reduced by cell preincubation for 24 h with 30 ng/ml IFN-β (Fig. 35).

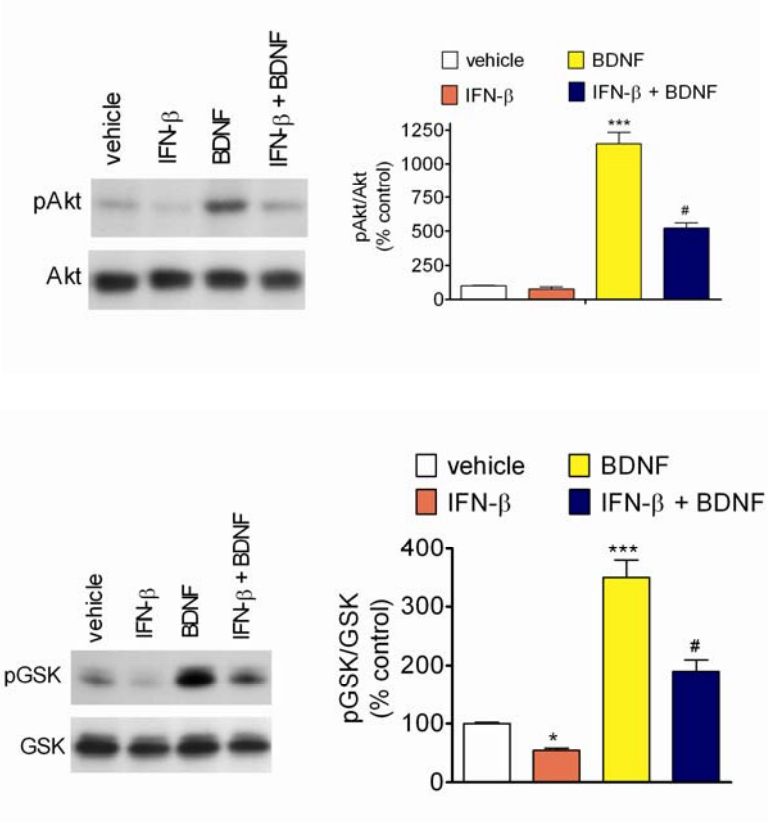


Fig. 35

IFN-β inhibits BDNF-stimulated Akt and GSK-3β phosphorylation in differentiated SH-SY5Y cells. SY-SY5Y cells were differentiated by exposure to 10 μM retinoic acid for 6 days. Cells were treated with either vehicle or IFN-β (30 ng/ml) for 24 h and then exposed to either vehicle or 0.9 nM human BDNF for 5 min. Cell lysates were then assayed for phospho-(Thr308)-Akt and phospho-(Ser9)-GSK-3β. Values are the mean ± SEM of three experiments. * $p < 0.05$, *** $p < 0.001$ vs control. # $p < 0.05$ vs BDNF alone.

Besides regulating the PI3K/Akt/GSK-3β pathway, BDNF has been reported to promote the phosphorylation of the ERK1/2 MAPK in SH-SY5Y cells (Encinas *et al*., 1999). Cell exposure to IFN-β for 24 h was found to significantly decrease the stimulation of ERK1/2 elicited by acute treatment with BDNF (Fig. 36).

Previous studies have shown that, as compared to RA and PMA alone, differentiation of SH-SY5Y cells with sequential RA and PMA treatment exhibited the highest level

of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis (Presgraves *et al.,* 2004). In RA/PMA differentiated SH-SY5Y cells, BDNF (0.9 nM) caused an increase in the level of TH phosphorylation at Ser31 and this effect was curtailed by cell pre-treatment with IFN-β (Fig 37).

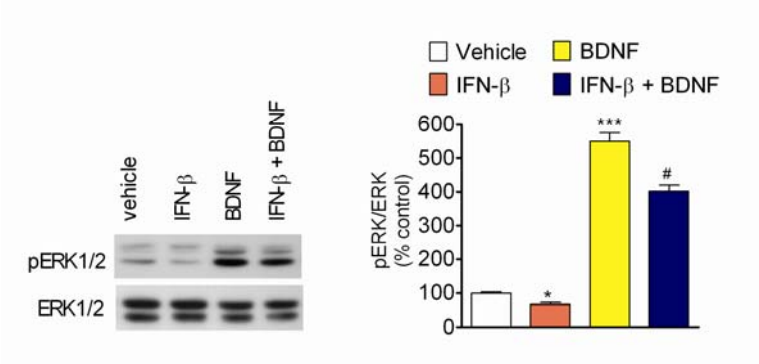


Fig. 36

IFN-β attenuates BDNF-induced stimulation of ERK1/2 phosphorylation in differentiated SH-SY5Y cells. RA-differentiated SH-SY5Y cells were treated with either vehicle or IFN-β (30 ng/ml) for 24 h and then exposed to either vehicle or 0.9 nM human BDNF for 5 min. Cell lysates were then assayed for phospho-ERK1/2. Values are the mean ± SEM of three experiments. * $p < 0.05$, *** $p < 0.001$ vs control. # $p < 0.05$ vs BDNF alone.

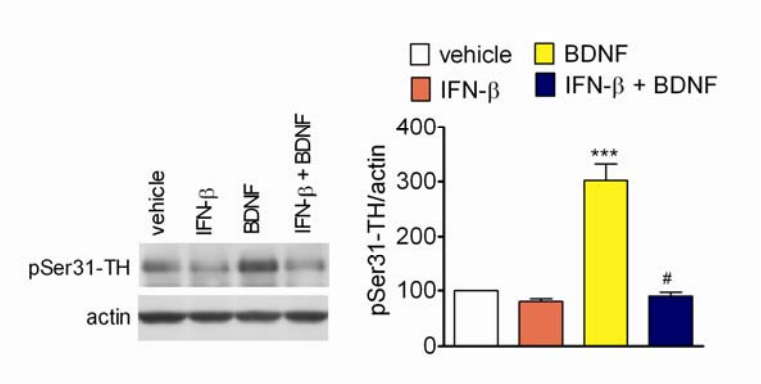
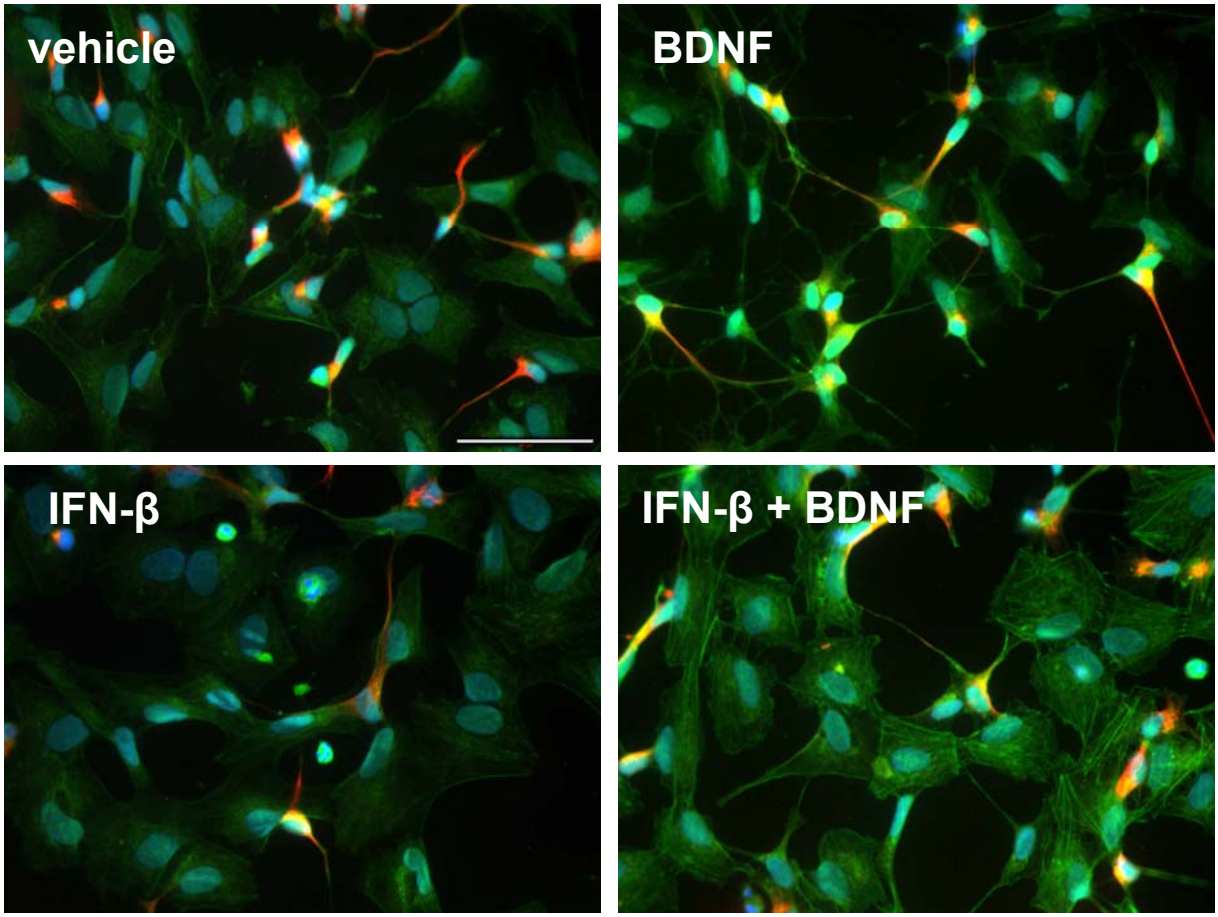


Fig. 37

SH-SY5Y were differentiated by sequential treatment with RA for two days and PMA for two additional days. Cells were incubated for 24 h with either vehicle or IFN-β (30 ng/ml) and then exposed to either vehicle or 0.9 nM BDNF for 5 min. Values are the mean ± SEM of three experiments.*** $p < 0.001$ vs control, # $p < 0.001$ vs BDNF alone.

In RA-differentiated SH-SY5Y cells, prolonged treatment with BDNF has been reported to induce a significant increase in the number of cells exhibiting neuritic processes (Encinas *et al*., 1999). To investigate whether exposure to type I IFNs could affect BDNF-stimulated neuritogenesis, SH-SY5Y cells were differentiated with RA and then incubated for 48 h with 2 nM BDNF alone or in association with IFN-β (30 ng/ml). Cells were then stained with FITC-conjugated phalloidin and NF160/200. In line with previous observations (Encinas *et al*., 1999), it was found that, as compared to control cells, BDNF-treated cells exhibited a higher number of neurites, which were also longer and branched (Fig. 38). Moreover, BDNF-treated cells displayed smaller cell bodies with an oval shape and appeared to spread over the dish surface. The co-addition of IFN-β counteracted these morphological changes, causing the cells to become flattened with fewer and shorter processes (Fig. 38).



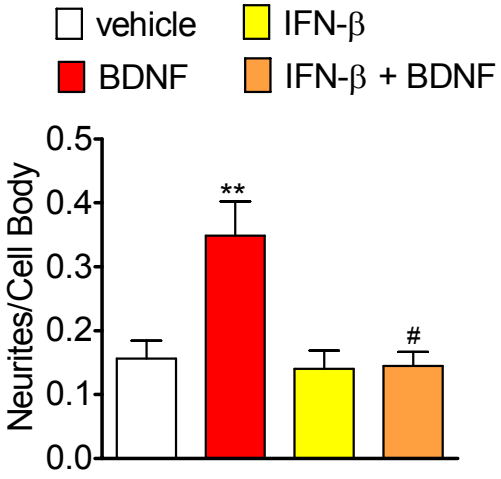


Fig. 38

IFN-β inhibits BDNF-induced neuritogenesis in SH-SY5Y cells. RA-differentiated SH-SY5Y cells were incubated with either vehicle, 2 nM BDNF, 30 ng/ml IFN-β or the combination of IFN-β and BDNF for 48 h. Cells were then stained with anti-neurofilament 160/200 (red), FITC-conjugated phalloidin (green) and DAPI (blue). Data are representative of three similar experiments.
** $p < 0.01$ vs vehicle. # $p < 0.01$ vs BDNF alone.

3. Discussion

Previous studies examining the mechanisms of type I IFN neurotoxicity have mainly postulated indirect effects of the cytokines mediated by an action on either peripheral organs or glial cells. In the present study we show that type I IFNs can act on neuronal cells to promote apoptotic death, thus indicating that neurons may constitute a direct cellular target of the neurotoxic action of these cytokines. The pro-apoptotic effects of type I IFNs were observed in primary cultures of mouse cortical neurons and in SH-SY5Y neuroblastoma cells. Conversely, under similar experimental conditions, IFN-β failed to cause cell death in the HeLa cell line, which also expresses functional IFNAR. This observation suggests that neuronal cells are particularly sensitive to the cytotoxic effects of type I IFNs.

The pro-apoptotic action of IFNs appeared to be dependent on the regulation of different signaling pathways, which are known to govern cell survival through transcriptional, translational and post-translational mechanisms.

3.1 Role of JAK-STAT signaling in IFN-induced apoptosis.

In neuronal cells, type I IFNs behaved as effective stimulators of JAK-STAT signaling. In SH-SY5Y cells IFN-α and -β elicited a rapid induction of Tyk2, STAT1, STAT3 and STAT5 tyrosine phosphorylation at concentrations in the picomolar range, indicating that the cells possess a type I IFN receptor with high affinity for the cytokines. A robust increase of STAT1 and STAT3 phosphorylation by IFN-β was similarly observed in mouse primary neurons. In SH-SY5Y cells the expression of a specific type I IFN receptor was further supported by the ability of an anti-IFNAR2 neutralizing antibody to completely block STAT1 phosphorylation induced by IFN-β.

Impairment of JAK activity by the potent pan-JAK inhibitor P6 attenuated Tyk2, STAT1 and STAT3 tyrosine-phosphorylation and ISRE transcriptional activity. Moreover, P6 treatment prevented IFN-β-induced caspase activation in both SH-SY5Y cells and mouse primary neurons. Among the STAT family members, STAT1 has been shown to promote anti-proliferative effects and different forms of cell death in response to cytokines through transcription-dependent and –independent processes (Kim and Lee, 2007). On the other hand, in various cell types STAT3 and STAT5 have generally been reported to induce the expression of anti-apoptotic and pro-survival genes (Schindler *et al*., 2007) and in THP-1 human monocytic cells STAT3 has been found to antagonize STAT1 dependent transcription in response to type I IFNs (Ho and Ivashkiv, 2006). Although in SH-SY5Y cells type I IFNs acutely stimulated tyrosine-phosphorylation of STAT1, STAT3 and STAT5, prolonged treatment with IFN-β caused a more robust induction and a more persistent phosphorylation of STAT1 than STAT3 or STAT5. Thus, the induction of apoptosis in SH-SY5Y cells appeared to be associated with an imbalance between STAT1, STAT3 and STAT5 activation states.

3.2 Involvement of PKR

PKR is one of the most characterized death-modulating protein induced by type I IFNs through the activation of STAT1. PKR activated by dsRNAs generated during viral infections, or by direct interaction with the activator protein PACT/RAX, undergoes dimerization and autophosphorylation. Activated PKR then phosphorylates eIF-2α, thereby inhibiting mRNA translation, cell growth or viral replication (Gil and Esteban, 2000). In SH-SY5Y cells, IFN-β caused a time-

dependent increase in the level of PKR protein expression, which was dependent on RNA synthesis and JAK-STAT signaling, as indicated by the sensitivity to P6 and STAT1 siRNA treatments. The enhanced PKR levels were associated with enhanced activity leading to an increased phosphorylation of eIF-2α. Inhibition of PKR prevented IFN-β-induced activation of the caspase proteolytic cascade, indicating that PKR played a major role in the IFN-β-triggered apoptotic process. Previous studies on PKR-induced apoptosis have clearly established that eIF-2α phosphorylation is an essential step, but the precise connection between PKR activity and cell death is still unknown. Two main pathways linking PKR to cell death have been hypothesized: one mediated by activation of the transcription factor NF-κB leading to increased expression of pro-apoptotic proteins, such as Fas, Bax and p53, and the other involving caspase-8 activation through the death receptor FADD (Gil and Esteban, 2000). Whether these mechanisms contribute to IFN-β-induced apoptosis in SH-SY5Y cells remains to be investigated. However, in agreement with other studies (Hopkins-Donaldson *et al*., 2000) pro-caspase-8 and its proteolytic fragments were not detected in SH-SY5Y cells under basal conditions and following treatment with IFN-β or TNF-α, thus making the involvement of this caspase pathway unlikely.

An additional mechanism of PKR-induced apoptosis may involve the transcriptional activation of the gene for C/EBP (CCAAT/enhancer binding protein) homologous protein (CHOP), also known as growth arrest and DNA damage inducible gene 153 (GADD153), which is a component of the endoplasmic reticulum stress (Oyadomari and Mori, 2004). Although phosphorylation of eIF-2α causes a general attenuation of translation, this condition allows the efficient translation of the activating transcription

factor (ATF) 4 mRNA, since this mRNA contains small upstream open-reading frames (uORF) within the 5'-untranslated region, which allows to overcome the blockade induced by phosphorylated eIF-2α. Once translated, ATF4 migrates to the nucleus where it binds to the cis-acting elements AARE 1 and AARE2, which are known to regulate the transcription of CHOP. CHOP is a pro-apoptotic 29 kDa protein composed of two known functional domains, a N-terminal transcriptional activation domain and a C-terminal basic-leucine zipper (bZIP) domain formed by a basic amino acid rich DNA-binding region followed by a leucine zipper dimerization motif. The bZip domain allows the formation of heterodimers with other C/EBP family member of transcription factors that prefer to form homodimers, As a consequence, CHOP-C/EBP heterodimers cannot bind to C/EBP sites and regulate a variety of genes involved in cell differentiation and proliferation. Moreover, overexpression of CHOP leads to a decrease of the anti-apoptotic protein Bcl-2 (McCullough *et al*., 2001) and to translocation of the pro-apoptotic protein Bax from the cytosol to the mitochondria (Gotoh *et al*., 2004). Thus, CHOP induction triggered by enhanced eIF-2α phosphorylation by PKR may contribute to IFN-induced neuronal death by enhancing the mitochondrial apoptotic pathway (Fig. 39).

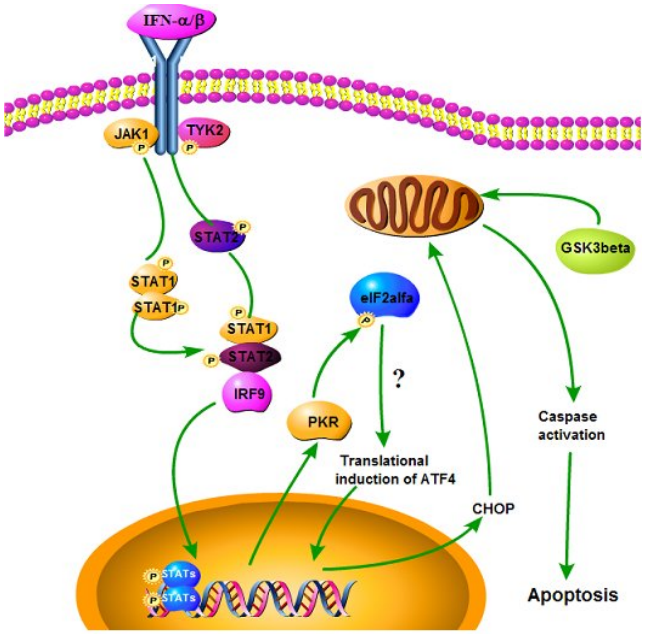


Fig. 39

Possibile involvement of the pro-apoptotic CHOP in PKR-induced neuronal death.

3.3 Role of p38 MAPK in IFN-induced neuronal apoptosis

Although considered as an ancillary pathway of IFN signaling, the p38 MAPK has been reported to play a critical role in IFN cellular actions. In different cell types, treatment with the p38 MAPK inhibitor SB203580 or overexpression of a dominant-negative p38 MAPK mutant inhibited IFN-α-dependent gene transcription via ISRE and GAS elements (Uddin *et al*., 1999, 2000; Goh *et al*., 1999). Moreover, p38 MAPK activity has been shown to be required for IFN-induced antiviral activity (Goh *et al.,* 1999), growth inhibitory effects on human hematopoiesis (Verma *et al.,* 2002) and antiproliferative effects in leukaemia cells (Mayer *et al.,* 2001).

The present study shows that in SH-SY5Y cells IFN-β induced a significant increase of the phosphorylation of p38 MAPK, indicating that the cytokine can regulate this MAPK pathway also in neuron-like cells. The enhanced p38 MAPK phosphorylation became significant after a lag time of about 3 h, suggesting that the coupling of IFN-activated IFNAR to p38 MAPK stimulation was slow and possibly required a multistep

process. Cell treatment with P6 prevented IFN-β-induced p38 MAPK phosphorylation, a finding in line with previous studies reporting that JAK activity is required to trigger the signaling cascade leading to p38 MAPK activation (Platanias, 2003). Cell treatment with SB203580 prevented IFN-β-induced caspase activation and DNA fragmentation, indicating that p38 MAPK activity was involved in the apoptotic process. The mechanism(s) by which p38 MAPK may promote apoptosis in IFN-treated SH-SY5Y cells have not been investigated. As mentioned above, p38 MAPK has been found to facilitate IFN-dependent gene transcription and, therefore, may potentiate STAT-dependent mechanisms of apoptosis. However, in the present study blockade of p38 MAPK with SB203580 failed to prevent PKR induction by IFN-β (results not shown), suggesting that in SH-SY5Y cells p38 MAPK did not cause apoptosis through enhanced expression of PKR. In other cell types it has been reported that p38 MAPK can promote apoptosis through different mechanisms, including retinoblastoma protein phosphorylation (Yeste-Velasco *et al.,* 2009), Bax translocation to mitochondria (Capano and Crompton, 2006) and CHOP phosphorylation (Wang and Ron, 1996).

In the brain activation of p38 MAPK has been associated with neuroinflammation (Munoz and Ammit, 2010) and has been proposed to mediate κ-opioid dependent dysphoria (Bruchas *et al.,* 2007) and stress-induced depression (Duric *et al.,* 2010). In the rat embryonic raphe cell line RN46A and mouse midbrain and striatal synaptosomes p38 MAPK has been shown to mediate the stimulation of the serotonin transporter induced by IL6 and TNF-α (Zhu *et al.,* 2006), thus establishing a possible pathogenetic link between cytokines, p38 MAPK and depression (Miller and Raison, 2006).

3.4 Down-regulation of PI3K/Akt pathway

A large body of genetic and pharmacological evidence indicates that the PI3K/Akt signaling pathway is critical for cell growth and survival (Hennessy *et al.*, 2005). Previous studies have shown that short-term exposure to type I IFNs can activate PI3K through multiple mechanisms, including phosphorylation of STAT3, which functions as docking site for PI3K p85 regulatory subunit, IRS-1 tyrosine phosphorylation, and constitutive association of PI3K with IFNAR1 subunit of IFN receptor (van Boxel-Dezaire *et al.,* 2006). By stimulating PI3K/Akt signaling, type I IFNs have been found to promote the survival of primary astrocytes (Barca *et al.,* 2003), neutrophils (Wang *et al*., 2003) and B lymphocytes (Ruuth *et al*., 2001). Akt inactivates several pro-apoptotic factors, including the BAD protein, procaspase-9 and Forkhead transcription factors, and activates IkB kinase, leading to nuclear localization of the anti-apoptotic transcription factor NF-kB (Hennessy *et al.,* 2005). Akt phosphorylates GSK-3β on Ser9 and inhibits GSK-3β, a key enzyme in promoting the intrinsic apoptotic pathway (Pap and Cooper, 1998). In addition, acute PI3K activation has been proposed to promote mRNA translation of IFN-stimulated genes via activation of mammalian target of rapamycin (mTOR) (Kaur *et al*., 2008). In SH-SY5Y cells we observed that a brief exposure to IFN-β increased Akt phosphorylation, whereas a prolonged incubation with the cytokine led to down-regulation of Akt signaling, as indicated by the decreased phosphorylation and activation of Akt and reduced GSK-3β phosphorylation. Long-term treatment with IFN-β also inhibited Akt and GSK-3β phosphorylation in mouse primary neurons. The down-regulation of Akt signaling was likely due to a decreased activation of PI3K, as in SH-SY5Y cells IFN-β exposure led to a reduced responsivity to IGF-1, which

stimulates Akt by activating receptors coupled to PI3K. Cell transfection with constitutively active Akt reduced IFN-β-induced apoptosis, indicating that IFN-inhibited Akt signaling affected neuronal survival. Thus, it is possible that the down-regulation of PI3K/Akt activity may promote neuronal death by facilitating the activation of the intrinsic apoptotic pathway.

The precise molecular mechanisms mediating the IFN-β-induced down-regulation of PI3K/Akt signaling remain unknown. In SH-SY5Y cells we have found that prolonged IFN-β treatment led to a reduced tyrosine phosphorylation of IRS-1, which is a critical component of PI3K recruitment and activation process by both IFN and growth factor receptors (Uddin *et al*., 1995). This change could therefore have been involved in down-regulation of PI3K/Akt signaling. The altered tyrosine phosphorylation state of IRS-1 could have been a consequence of a decreased JAK activity (Burfoot *et al.,* 1997), possibly due to the action of suppressor of cytokine signaling family of proteins, which are known to be induced by IFNs and to provide a negative feedback loop on IFN signaling by direct JAK inhibition (Shuai and Liu, 2003). However, since IFN treatment also led to a decreased IRS-1 phosphorylation in response to IGF-1, additional mechanisms including protein tyrosine phosphatases (PTPs) could have been involved. Several PTPs known to promote IRS-1 dephosphorylation, such as PTP1B and SHP-2 (Goldstein *et al.,* 2000), also participate in the regulation of strength and duration of IFN signaling (Shuai and Liu, 2003). Interestingly, it has recently been reported that IRS-1 is dephosphorylated by the protein tyrosine phosphatase PTPL1, which causes apoptosis and inhibits PI3K/Akt pathway (Dromard *et al.,* 2007). Further studies are required to investigate whether this or other PTPs is involved in IFN-induced neuronal cell death.

3.5 Role of GSK-3 in IFN-induced neuronal damage.

As mentioned above, along the PI3K/Akt pathway GSK-3 works as a major regulator of cell survival and an impaired inhibition of GSK-3β via phosphorylation at Ser9 has been associated with mood disturbances, schizophrenia and neurodegenerative disorders (Beurel and Jope, 2006; Prickaerts *et al*., 2006; Rowe *et al*., 2007; Hooper *et al*., 2008; Li and Jope, 2010). Increasing evidence also indicates that GSK-3 is a key regulator of neurodevelopmental processes, including neurogenesis, neuronal migration, neuronal polarization and axon growth and guidance (Hur and Zhu, 2010). Moreover, inhibition of GSK-3 signaling is required for hippocampal long-term potentiation, a synaptic model of learning and memory (Hooper *et al.,* 2007).

In either primary cultures of mouse cortical neurons and SH-SY5Y cells long-term exposure to type I IFNs led to a reduced phosphorylation of GSK-3β at Ser9, indicating an enhanced kinase activity. Moreover, the results obtained with the two inhibitors SB216763 and AR A014418 highlight a crucial role of GSK-3 in neuronal death caused by IFN exposure. In fact, blockade of activity prevented the IFN-induced activation of caspase cascade and significantly reduced IFN-stimulated DNA fragmentation, indicating a close involvement in the apoptotic outcome.

GSK-3β has previously been found to promote amyloid-β-induced neurotoxicity (Takashima *et al.,* 1993), and its overexpression in the rat pheocromocytoma cell line PC12 caused apoptosis (Pap and Cooper, 1998). In addition, GSK-3β has also been involved in neuronal apoptosis induced by trophic withdrawal (Hetman *et al*., 2000; Mishra *et al.,* 2007; Wang *et al*., 2009), the human immunodeficiency virus type 1 regulatory protein Tat (Maggirwar *et al*., 1999) and 6-hydroxydopamine (Li *et al.,* 2011).

Different mechanisms may mediate the pro-apoptotic effects of GSK-3β. This protein kinase has been reported to regulate cell activity and survival by controlling both transcriptional and non-trascriptional events. GSK-3β phosphorylates and affects the function or stability of various transcription factors that control genes governing cell fate and differentiation, such as p53, β-catenin, heat-shock factor 1 (HSF-1), myc, NF-κB and cyclic AMP responsive element binding (CREB) protein (Beurel and Jope, 2006). With regard to neuronal apoptosis, both inhibition of HSF-1 and CREB has been proposed to play a role. Once activated, HSF-1 induces the expression of heat shock proteins that detect and chaperone denaturated and misfolded proteins. Similarly, inhibition of CREB transcriptional activity may participate in the pro-apoptotic effect of GSK-3β, as CREB has a critical role in promoting neuronal survival (Walton and Dragunow, 2000). Moreover, CREB is involved in many central functions negatively affected by IFN administration, such as mood regulation (Pandey, 2004) and cognitive processes (Silva *et al*., 1998; Benito and Barco, 2010).

More recently, GSK-3β has been reported to cause neuronal apoptosis through phosphorylation and inactivation of the transcription factor myocyte enhancer factor 2D (MEF2D), which promotes neuronal survival (Wang *et al.,* 2009).

GSK-3 can also induce apoptosis through non-transcriptional mechanisms, including phosphorylation of the microtubule-associated protein tau and consequent microtubule destabilization (Johnson and Bailey, 2002), the pro-apoptotic protein Bax (Linseman *et al.,* 2004) and the mitochondrial voltage-dependent anion channel (VDAC) involved in the control of mitochondrial membrane potential. The GSK-3-induced phosphorylation of VDAC prevents its association with hexokinase II and favours the binding of pro-apoptotic Bcl-2 family proteins with a consequent

enhancement of mitochondrial permeability (Pastorino *et al.,* 2005). Moreover, GSK-3β has been proposed to induce neuronal apoptosis through phosphorylation and activation of the mixed lineage kinase 3 (MLK3), which triggers the JNK pathway (Mishra *et al.,* 2007).

Inhibition of GSK-3 with AR A014418 was found to prevent the increased expression of PKR caused by long-term IFN-β treatment, suggesting that GSK-3 regulates IFN transcriptional activity. A recent study has shown that in mouse primary astrocytes, microglia and macrophage-derived RAW264.7 cells, IFN-γ/LPS-induced STAT3 tyrosine phosphorylation was greatly reduced by GSK-3 inhibitors and that GSK-3β bound to STAT3 and associated with IFN-γ receptor (Beurel and Jope, 2008). Thus, it is possible that the protective effects of GSK-3 inhibitors against IFN-β-induced apoptosis may also result from a reduced STAT activation. It remains to be investigated whether in SH-SY5Y cells GSK-3 regulates STAT1 phosphorylation through a direct interaction with IFNAR.

Interestingly, antidepressant drugs have been reported to inhibit GSK-3β by increasing Ser9 phosphorylation (Beurel and Jope, 2006; Prickaerts *et al*., 2006; Rowe *et al*., 2007). Antidepressants are also effective in preventing type I IFN-induced depression (Raison *et al*., 2005). It is therefore possible that inhibition of IFN-enhanced GSK-3β activity may contribute to the beneficial effects of these drugs against the mood disturbances associated with IFN therapy. This hypothesis requires experimental validation.

3.6 IFN-induced inhibition of BDNF signaling and neurotrophic effects.

The long-term exposure of RA-differentiated SH-SY5Y cells to type I IFNs was found to markedly affect the ability of BDNF to regulate intracellular signaling and neuronal differentiation. The activation of Akt and the increased phosphorylation of GSK-3β at Ser9, two events associated with BDNF-stimulated neuronal survival and antidepressant effects (Encinas *et al.,* 1999; Mai *et al.,* 2002) were significantly reduced in cell pre-exposed to IFN-β. The inhibitory effects of IFN-β were not limited to BDNF-activated PI3K/Akt signaling but also involved BDNF stimulation of ERK1/2 MAPK pathway, suggesting a wide suppressant effect on Trk signaling. In SH-SY5Y cells, ERK1/2 activation has been found to be necessary for BDNF-induced neuritogenesis (Encinas *et al*., 1999). It was found that IFN-β significantly reduced BDNF-stimulated neurite outgrowth, indicating that the inhibition of ERK1/2 activation had relevant consequences on neuronal differentiation induced by the neurotrophin.

Another important outcome of ERK1/2 inhibition may be represented by the reduction of BDNF-stimulated TH phosphorylation at Ser31 in cell pre-exposed to IFN-β. Activation of the ERK1/2 pathway has been reported to induce TH phosphorylation at Ser31 and to stimulate catecholamine synthesis (Haycock *et al*., 1992; Halloran and Vulliet, 1994; Bobrovskaya *et al*., 2001; Lindgren *et al.,* 2002). As impaired nor-adrenergic transmission may be associated with depression (Delgado, 2000), the IFN-induced inhibition of BDNF-stimulated TH phosphorylation and the possible associated reduction of catecholamine synthesis may contribute to the mood disturbances elicited by type I IFNs.

A recent study has proposed that stress stimuli may impair ERK1/2 activity through p38 MAPK-dependent induction of the MAPK phosphatase-1 (MKP-1), a tyrosine and serine/threonine phosphatase that targets MAPK (Duric *et al*., 2010). In future studies, it will be important to assess whether IFN-induced inhibition of ERK1/2 signaling is consequent to p38 MAPK activation and involves an enhanced expression of MKP family members.

Conclusions

In conclusion, the present study provides the first demonstration that type I IFN can act directly on neuronal cells to impair cell survival through multiple mechanisms, including stimulation of JAK-STAT signaling, PKR induction and activation, enhanced p38 MAPK activity, down-regulation of the PI3K/Akt pathway and attenuation of BDNF signaling (Fig 40). In SH-SY5Y cells, these intracellular signals may cooperate to promote mitochondria damage, thereby triggering cytochrome C release, activation of caspase cascade and apoptosis. Moreover, the antagonism of neurotrophin action may lead to impairment of neuritogenesis and reduced catecholamine neurotransmission. Neuronal apoptosis and altered synaptic plasticity are considered relevant neuropathological substrates of several neurodegenerative and neuropsychiatric disorders (Mattson, 2000; McKernan *et al*, 2009). It is therefore possible that these functional and structural alterations of the neuronal network may contribute to the pathogenesis of the central neurotoxic effects of type I IFNs.

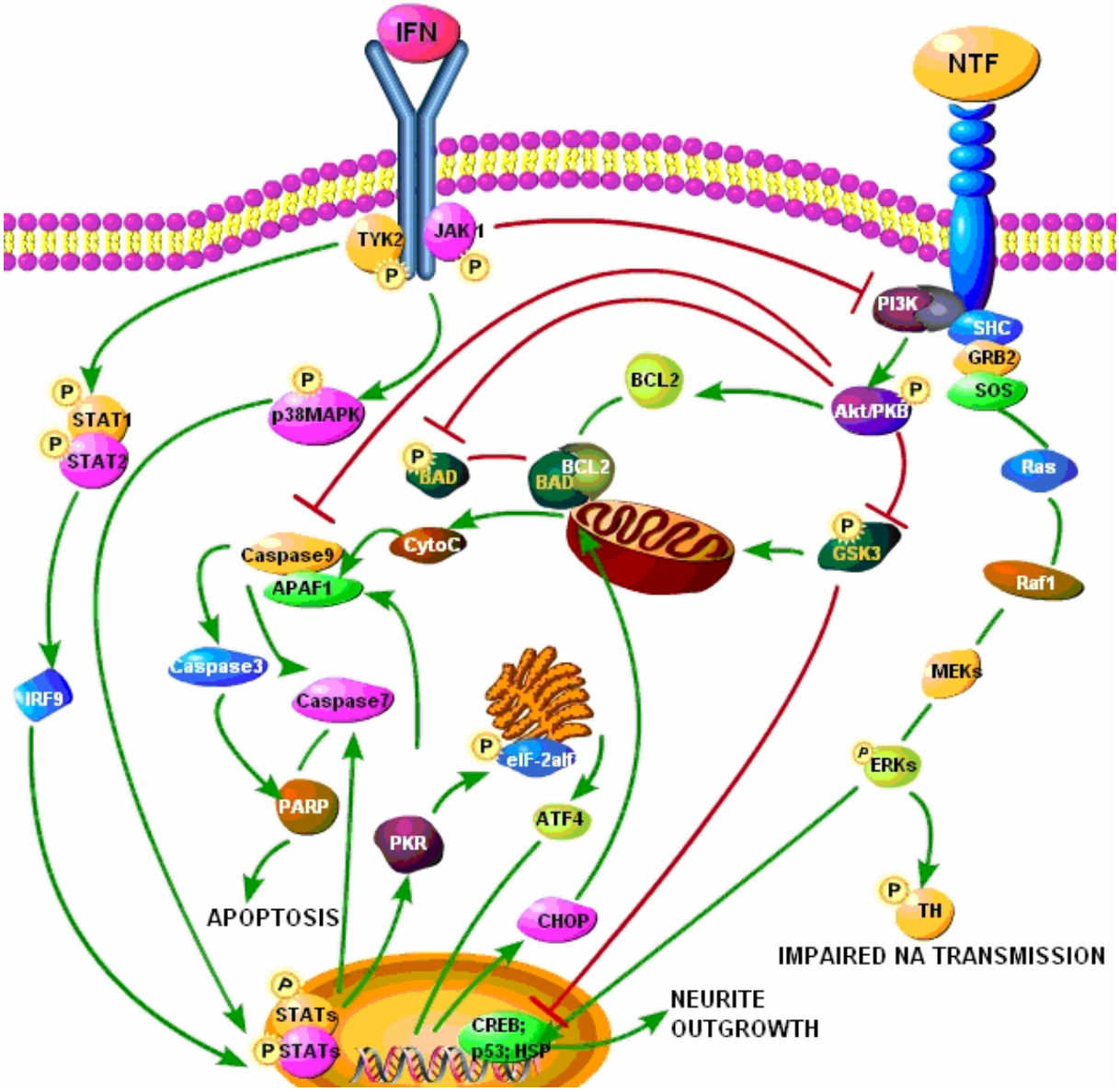


Fig. 40

Proposed intracellular pathways mediating neuronal damage induced by type I IFNs. The green and red lines arrows indicate stimulatory and inhibitory pathways, respectively. NA., nor-adrenaline, NTF, neurotrophic factor.

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