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ARTICLE

N-acetyl-cysteine prevents toxic oxidative effects induced by IFN-*α* in human neurons

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Abstract

Currently IFN-*α* is widely used for effective treatment of viral infections and several malignancies. However, IFN-*α* can cause neuropsychiatric disturbances and mental impairments, including fatigue, insomnia, depression, irritability and cognitive deficits. Molecular and cellular mechanisms leading to such side-effects are still poorly understood. Neurons seem to be an important target in mediating cellular effects induced by exposure to this cytokine, but so far little is known about IFN-*α*-induced effects on these cells. We have investigated the ability of IFN-*α* (2–100 ng/ml) to induce damage and toxicity to the human neuroblastoma SH-SY5Y cell line, commonly used for studying such phenomena, and the mechanisms underlying these effects. After 24 h treatment, IFN-*α* increased mitochondrial activity, whereas cell density was reduced in a dose- and time-dependent manner. This effect did not depend on reduced cell proliferation, but rather the activation of apoptosis, as revealed by an increased Bax:Bcl-2 mRNA ratio after 72-h IFN-*α* exposure. At this time-point, IFN-*α* also reduced the expression of the brain-derived neurotrophic factor gene, and induced an increase in reactive oxygen species (ROS). A co-treatment with *N*-acetyl-cysteine (NAC; 5 mM), a potent antioxidant and mitochondrial modulator, was able to counteract all of these IFN-*α*-induced effects. These findings demonstrated that IFN-*α* induces neurotoxicity and apoptosis that is, in part, very likely due to mitochondrial damages and production of ROS. We suggest that NAC, already tested for the treatment of psychiatric disorders, may be useful to prevent IFN-*α*-induced central side-effects in a safe and effective way.

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Introduction

Interferon-*α* (IFN-*α*) is used for the therapy of different malignancies, including melanoma and renal carcinoma, and is the gold standard for treating hepatitis C virus (HCV) infections. The World Health Organization estimated that in 1999 about 3% of the world's population (i.e. about 170 million people) are living with HCV (Sy and Jamal, 2006) and each year >350,000 subjects die from HCV-related conditions (Averhoff et al., 2012). For patients infected with most HCV genotypes, the combination of the pro-inflammatory cytokine IFN-*α* and the antiviral drug ribavirin (RBV) represents the standard-of-care treatment. Although this regimen is quite effective for treating HCV infection, leading to sustained virological response, it has long been observed that IFN-*α* plus RBV have some severe side-effects that can limit the use of this combination in a significant number of HCV patients (Wartelle-Bladou et al., 2012).

Neuropsychiatric disturbances and mental impairments are among the most common and serious complications associated with IFN-*α* treatment (Baraldi et al., 2012; Schaefer et al., 2012; Zunszain et al., 2012b). Depressed mood, fatigue, insomnia, anhedonia, irritability, cognitive deficits, mania, delirium, psychotic symptoms and even suicidal thoughts, have been observed during this therapy. Indeed, ⩽ 70% of patients

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treated with IFN-α experience mild-to-moderate depressive syndromes (Schaefer et al., 2012), and ~30% have major depression (Hepgul et al., 2012). IFN-α-induced psychiatric symptoms not only strongly affect patient's quality of life but may also significantly compromise therapeutic response (Maddock et al., 2005; Leutscher et al., 2010). Preventing onset of neuropsychiatric symptoms in IFN-α-treated patients is therefore an important medical need, and such a goal has to be achieved using methods that do not interfere with the efficacy of the antiviral treatment. However, a relevant obstacle is that the molecular mechanisms responsible for IFN-α-induced depression are still largely unknown.

It is now well recognized that both systemic and intra-cerebral IFN-α administration induce a variety of central actions, ranging from changes in gene expression (Wang et al., 2008) to the development of a psychopathological phenotype in animal models and in humans (Felger et al., 2007; Hayley et al., 2012; Hepgul et al., 2012). This pro-inflammatory cytokine and its receptor (IFN-R) are present in the brain, where a fraction of the peripherally administered IFN-α may enter by diffusion through the blood–brain barrier (BBB; Vitkovic et al., 2000; Fioravanti et al., 2012) or by activating cells at the BBB to induce local production of IFN-α (Indraccolo et al., 2007). However, even if neurons seem to be an important target in mediating behavioural IFN-α-induced changes (Wang et al., 2008), so far little is known about the molecular effects induced by exposure to this cytokine on these cells.

The human neuroblastoma SH-SY5Y cell line possesses many characteristics of neurons, and is one of the most-used models for studying cellular events and mechanisms involved in neurotoxicity and neurodegeneration or even in neuroprotection (Yu et al., 2011; Emanuelsson and Norlin, 2012). Undifferentiated or differentiated SH-SY5Y cells have been used as a translationally-valid experimental model for several neurodegenerative and psychiatric disorders, including Parkinson's disease, Alzheimer's disease and depression, for understanding pathogenesis, mechanisms of disease progression and therapeutic targets (Donnici et al., 2008; Xie et al., 2010; Sutinen et al., 2012).

Thus, in order to test whether human recombinant IFN-α was able to induce direct neurotoxicity, and the mechanisms underlying these putative effects, we have used this *in vitro* model of human neurons, with the ultimate aim of identifying potential therapeutic targets for the management of IFN-α neuropsychiatric side-effects.

Materials and method

Cell culture

Human neuroblastoma SH-SY5Y cells were grown and maintained in complete medium [Dulbecco's modified Eagle's medium (DMEM) high glucose medium: Ham's nutrients mixture F-12 (1:1) containing 2 mM L-glutamine, 1% non-essential amino acids, 100 U/ml penicillin and 10 μg/ml streptomycin, supplemented with 10% foetal bovine serum (FBS)], at 37 °C in a humidified atmosphere of 5% CO_2 in air. All reagents used to culture cells were purchased from Euroclone (Italy). Only cells between passages P18 to P25 were used. For the experiments, ~60% confluent cultures were harvested and maintained 24 h before the treatments in complete medium supplemented with 10% FBS or without FBS. We differentiated SH-SY5Y cells as neuron-like as previously described by Ojala et al. (2008). Briefly, cells were plated as 10^5 cells/well into 96-well plates in complete medium supplemented with 10% FBS and differentiated for 3 d with 10 μM all-trans retinoic acid (Sigma-Aldrich, USA). Then neuron-like differentiated SH-SY5Y cells were maintained for 4 d before treatment in complete medium without FBS, supplemented with 50 ng/ml human recombinant brain-derived neurotrophic factor (BDNF; Immunological Sciences, Italy). Human glioblastoma-astrocytoma U-87 MG cells were grown in DMEM with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

Cell treatments

Following seeding (after 24 h), cells were treated with recombinant human cytokines: IFN-α (2, 20 or 100 ng/ml; IFN-α2; PeproTech); interleukin (IL)-1β (10 ng/ml, PeproTech); IL-18 (100 ng/ml, ProSpec) for different time periods depending on the experiment (6, 24, 48 and 72 h). Cells were also treated for 72 h with *N*-acetyl-cysteine (NAC, 5 mM; Sigma-Aldrich) alone or in association with IFN-α (100 ng/ml corresponding to at least 18 UI/μl). Control samples received an equal amount of vehicle [sterile phosphate buffered saline (PBS)]. SH-SY5Y neuron-like differentiated cells were treated in complete medium supplemented with 50 ng/ml human recombinant BDNF.

Quantitative and qualitative PCR analysis

Cells were plated at a density of 10^6 cells/well on 6-well plates in 1 ml complete medium containing 10% FBS. RNA preparation and DNAse treatment were performed as previously described (Alboni et al., 2011a). Total RNA was reverse transcribed with a high

capacity cDNA reverse transcription kit (Applied Biosystems). Qualitative PCR analysis was carried out using GoTaq Flexi DNA polymerase (Promega Italia, Italy) starting from cDNA equivalent to 100 ng total RNA to demonstrate the expression of IFN-α, IFN-R_1 and IFN-R_2 mRNAs in SH-SY5Y cells by using specific primer pairs. Ubiquitin C (UBC) mRNA expression was used as a control. Real-time PCR was performed in ABI PRISM 7900 HT (Applied Biosystems) using Power SYBR Green mix (Applied Biosystems) by using specific primer pairs for B-cell CLL/lymphoma 2 (Bcl-2)α, Bcl-2-associated X protein (Bax)-β and BDNF (exon IX; see Supplementary Table S1 for primer sequences). Each sample was normalized to the expression of the housekeeping gene UBC. Cycle threshold (Ct) value was determined by the SDS software 2.2.2 (Applied Biosystems) and was used to calculate mRNA fold changes using the ΔΔCt method as described elsewhere (Alboni et al., 2011b).

Cytotoxicity assays

Thiazolyl Blue Tetrazolium Bromide assay

One day after plating on 96-well plates (10^4 SH-SY5Y cells/well), cells were treated with human recombinant cytokines in complete medium with 10% FBS or without serum. Cells were maintained at 37 °C in a humidified 5% CO_2/air atmosphere for 24, 48 or 72 h then cells were incubated for 4 h with a Thiazolyl Blue Tetrazolium Bromide (MTT; Sigma Aldrich, USA) solution (5 mg/ml). The formazan formed was dissolved in 150 μl acid isopropanol (0.1 N HCl in isopropanol) added to all wells and the absorbance was measured by a multiplate reader at 570 nm wavelength and at 620 nm as reference wavelength. All experiments were performed three to five times (n=8 for each experiment) in independent cultures. Results were expressed as percentage of control.

Crystal Violet staining

For the Crystal Violet (CV) test, cells were plated at a density of 10^5 SH-SY5Y cells/well on 96-well plates in complete medium containing 10% FBS. One day after plating, cells were treated with human recombinant cytokines in complete medium with 10% FBS or without serum. Cells were maintained at 37 °C in a humidified 5% CO_2 /air atmosphere for 24, 48 or 72 h then after aspiration of culture medium, surviving cells were fixed with 1% glutaraldeide (Sigma-Aldrich) and stained with 0.5% CV in 95% ethanol for 20 min at room temperature. Then plates were washed and dried, 100 μl 10% acetic acid solution was added to all wells and the absorbance at 570 nm wavelength was measured spectrophotometrically for quantitative evaluation. All experiments were performed three to five times (n=8 for each experiment) in independent cultures. Results were expressed as percentage of control.

Morphological analysis

SH-SY5Y cells (5×10^4) grown on glass coverslips were treated 24 h after plating with IFN-α alone or in combination with NAC for 1 or 3 d and then washed with PBS, fixed in 4% paraformaldehyde (PFA) in PBS. Coverslips were mounted with Mowiol solution, cells were examined and images were acquired through a digital AxioCam HRc colour camera mounted on a AxioScope 40 (Zeiss) using the AxionVision program.

Cell proliferation

5-Bromo-2′-deoxyuridine labelling

SH-SY5Y cells were incubated for 2 h with 10 μM 5-bromo-2′-deoxyuridine (BrdU), rinsed in PBS and fixed in freshly-made 4% PFA in PBS for 30 min at 4 °C and rinsed three times with PBS-Triton X-100 1%. The cells were incubated in HCl (1 N) for 10 min on ice, in HCl (2 N) for 10 min at room temperature and for 20 min at 37 °C. Acid was neutralized by washing with 0.1 M borate buffer at pH 8.5 for 12 min at room temperature. After washing, cells were incubated for 1 h at room temperature with blocking buffer (BB: 5% FBS; glycine 1 M; 1% Triton X-100 in PBS) and finally incubated overnight with the monoclonal anti-BrdU antibody (1:30; Becton-Dickinson, USA) in BB. Cells were incubated for 1 h at room temperature with FITC-conjugated goat anti-mouse Ab in BB (1:100; Santa Cruz Biotechnology, USA). After washing once with PBS for 5 min at room temperature, the cells were incubated with PBS-Tween 20 0.1% (DAPI; 100 ng/ml; Sigma-Aldrich) for 5 min. Before mounting in VECTASHIELD® mounting medium, coverslips were washed for 5 min in PBS and rinsed with deionized water. All treatment conditions were applied in duplicate. Images were acquired as described in the morphological analysis section. For quantification, five fields of identical size were analysed from two coverslips treated under the same experimental conditions, using identical microscope settings. The total number of cells was quantified by counting all DAPI-stained nuclei (n=5 replicates, 500 cells, 40× objective). The number of BrdU-stained nuclei was divided by the total number of DAPI-stained nuclei and expressed as a percentage of the total number of nuclei.

Apoptosis and oxidative stress

Staining with Hoechst

SH-SY5Y cells (10^5) cultured coverslips were fixed with 4% PFA in PBS (v/v) for 20 min at room temperature, washed with PBS, then exposed to 0.5% Triton X-100 for 5 min at room temperature and washed three times with PBS. Coverslips were exposed to 8 μg/ml Hoechst 33258 (Sigma Aldrich) dye in PBS for 5 min at room temperature, washed and mounted by using VECTASHIELD® mounting medium. Apoptosis was monitored by fluorescence microscopy (Carl Zeiss). Images were acquired as described in the morphological analysis section.

Terminal deoxynucleotidyl transferase dUTP nick end labelling test

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was performed starting from 3×10^5 SH-SY5Y cells, using the *In Situ* Cell Death Detection Kit, TMR red (Roche), following manufacturer's instructions. Cells were analysed and images were acquired as described in the morphological analysis section.

Flow cytometry

Intracellular hydrogen peroxide (H_2O_2) and mitochondrial anion superoxide ($mtO_2^{\bullet-}$) were detected by flow cytometry using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFH-DA), and MitoSOX Red mitochondrial superoxide indicator respectively. Intracellular fluorescences of H_2DCFH-DA and MitoSOX Red were in cells gated on the basis of physical parameters, to eliminate debris and dead cells, and thus determine H_2O_2 and $mtO_2^{\bullet-}$ levels selectively in viable cells. This approach is extremely useful to study reactive oxygen species (ROS) content in cells receiving different stimuli, and to analyse the relationship between oxidative stress and cell death (Cossarizza et al., 2009). Briefly, cells were treated as described earlier, trypsinized, resuspended in complete medium, and incubated with 5 μM MitoSOX at 37 °C. After centrifugation, cells were incubated with 2 μM H_2DCFH-DA in PBS at 37 °C. Cells were resuspended in PBS and, before acquisition, 0.1 μM TO-PRO-3 was added to assess cell viability and exclude dead cells.

Apoptosis was detected combining Annexin-V Alexa Fluor 488 conjugate, and TO-PRO-3, as described (Troiano, 2007). All the relevant probes were from Life Technologies Corporation, USA.

Samples were acquired using a CyFlow ML flow cytometer (Partec GmbH, Germany); data from a minimum of 20 000 cells were analysed by FloMax (Partec) and FlowJo 9.4.11 (Treestar Inc., USA) software. Each experiment was repeated at least four times. Data obtained after staining with fluorescent probes represent the median of the net fluorescence value (±S.D.). The net fluorescence value was obtained by linearizing the fluorescence values from the logarithmic scale and subtracting the linearized median value of the blank from the median fluorescence value of the stained sample. Then, all data were compared to control samples, whose fluorescence was considered as 100.

Statistical analysis

Results are reported as mean±S.E.M. Data were analysed using the Statistical Package for Social Sciences, version 15.0 (SPSS Inc., USA). Statistical analysis was performed on three to five independent biological replicates (n=3–8 for each experiment) by either Student's t test, to compare means of two independent treatment groups, or an analysis of variance (ANOVA), followed by planned pairwise *post hoc* comparisons (Tukey's honestly significant difference) for multiple comparisons. All mean differences were considered statistically significant if $p<0.05$.

Results

IFN-α exposure induced cytotoxic effects against SH-SY5Y cells in a time- and dose-dependent manner

Because IFN-α effects are mediated by the binding to the IFN-α/β receptor (IFN-R), we first evaluated the expression of the two chains composing this receptor (IFN-R_1 and IFN-R_2). By using specific primer pairs we demonstrated that SH-SY5Y cells expressed both chains of the IFN-R, supporting the hypothesis that this clone is responsive to IFN-α-induced effects. We also demonstrated the expression of the IFN-α_2 mRNA (data not shown).

We then tested the hypothesis that IFN-α may induce toxic effects in this model of human neuron-like cells. IFN-α significantly affected cell density and viability in a time- (ANOVA univariate: $F_{2,215}$=14.000, $p<0.0001$ for CV test; $F_{2,221}$=17.026, $p<0.0001$ for MTT test) and dose- (ANOVA univariate: $F_{3,39}$=63.975, $p<0.0001$ for CV test; $F_{3,124}$=71.905, $p<0.0001$ for MTT test) dependent manner (Fig. 1). Specifically, after 72 h treatment with 100 ng/ml IFN-α, the number of cells was ∼50% lower (t=−11.945, $p<0.0001$) compared to the control group, while no effects were present after 24 or 48 h treatment (Fig. 1*a*). After 72 h treatment with IFN-α we also observed a strong

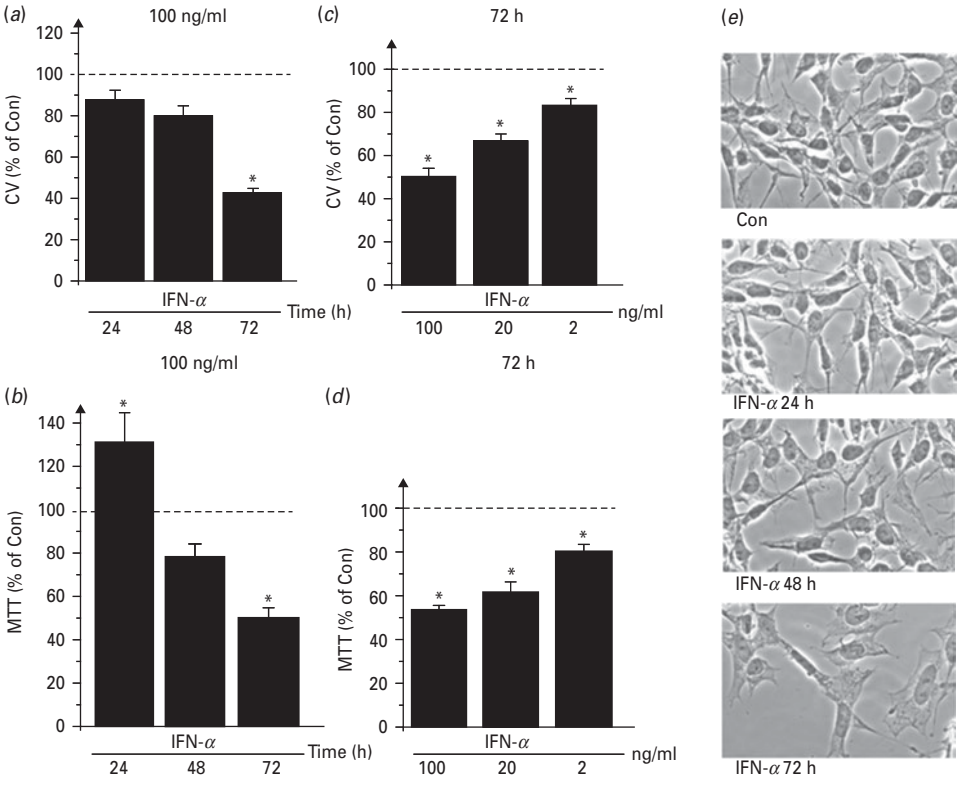

Fig. 1. Cytotoxity of interferon-α (IFN-α) against SH-SY5Y cells. IFN-α-induced effects on cell number [assessed using Crystal Violet (CV) staining] were time- (*a*) and dose- (*c*) dependent; in fact (*a*) IFN-α (100 ng/ml) did not affect SH-SY5Y cell number after a 24 h exposure but significantly reduced CV staining when compared to vehicle-treated control cells (Con), after 72 h treatment. Moreover, (*c*) all tested doses (2–100 ng/ml) were able to reduce the number of live cells after 72 h exposure but the higher effect was demonstrated for the highest dose tested (100 ng/ml). (*b*) Mitochondrial activity [assessed using the Thiazolyl Blue Tetrazolium Bromide (MTT) assay] was increased after 24 h exposure to IFN-α (100 ng/ml) and decreased after 72 h exposure. (*d*) IFN-α treatment (72 h) was able to reduce formazan production in a dose-dependent manner. Results are represented as mean±S.E.M. for $n=8$ for all (three to five) independent experiments. * Statistically significant difference from the control ($p<0.05$). (*e*) Examination of cells by light microscopy showed that IFN-α exposure for 72 h altered cell morphology causing process shortening and loss of neurites.

decrease in formazan production ($t=-15.420$, $p<0.0001$; Fig. 1*b*), which was likely dependent on the reduction of cell density (Fig. 1*a*, *b*). The MTT test demonstrated that mitochondrial metabolism increased 24 h after exposure to IFN-α ($t=4.437$, $p<0.0001$; Fig. 1*b*). After 72 h exposure, all tested doses (2, 20 and 100 ng/ml) were effective in reducing cell density and formazan production, with the strongest effect observed at the 100 ng/ml dose (−50% reduction, $p<0.0001$ for CV; −48%, $p<0.0001$ for MTT; Fig. 1*c*, *d*). Examination of cells by light microscopy showed that IFN-α exposure for 72 h altered cell morphology causing process shortening and loss of neurites (Fig. 1*e*).

We also tested the IFN-α-induced effects in neuron-like differentiated SH-SY5Y cells (Ojiala et al., 2008) and in serum deprivation conditions. We differentiated SH-SY5Y cells for 3 d 10 μM all-trans retinoic acid followed by 4 d 50 ng/ml human recombinant BDNF, and then we exposed neuron-like differentiated cells to IFN-α (100 ng/ml) for 24 or 72 h. Results in neuron-like differentiated SH-SY5Y cells (Fig. 2*a*, *b*) and in serum-free condition (Pirkmajer and Chibalin, 2011; Fig. 2*c*, *d*) were similar to those observed in undifferentiated cells in proliferating conditions (Fig. 1).

To test if the cytotoxicity induced by exposure to IFN-α was specific for cells with a neuronal phenotype, we performed similar experiments in U-87 MG cells, a human glioblastoma-astrocytoma cell line. IFN-α exposure did not significantly affect either the cell density or the formazan production at any time-point evaluated (24 and 72 h) in these cells (Fig. 2*e*, *f*).

Finally, to examine the specificity of IFN-α effect on cell density and viability in our experimental

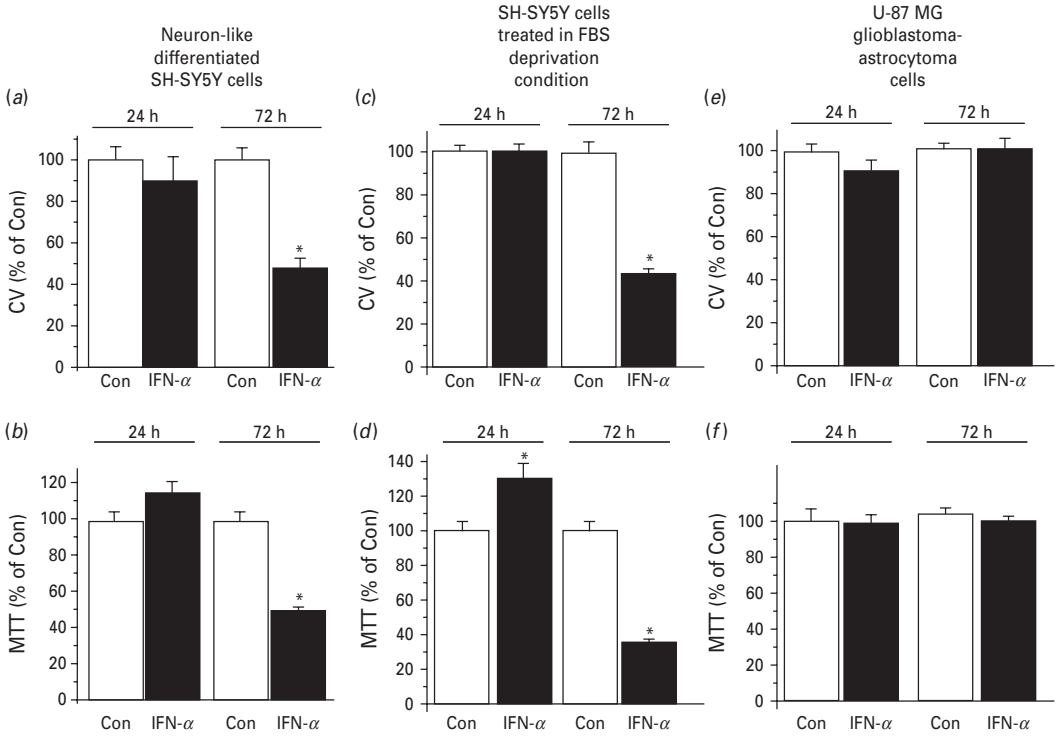

Fig. 2. Interferon-α (IFN-α)-induced cytotoxic effects against SH-SY5Y cells when maintained in serum deprivation condition and when neuroblastoma cells were differentiated to neurons with retinoic acid. IFN-α (100 ng/ml) did not induce cytotoxicity after 24 and 72 h exposure in a glioblastoma-astrocytoma cell line. IFN-α-induced effects on cell number [Crystal Violet (CV) staining; *a, c* and *e*] and mitochondrial activity [Thiazolyl Blue Tetrazolium Bromide (MTT) assay; *b, d* and *f*] following 24 or 72 h exposure against: (*a* and *b*) neuron-like differentiated SH-SY5Y cells [10 μM retinoic acid for 3 d; maintained for 4 d before treatment in complete medium without foetal bovine serum (FBS), supplemented with 50 ng/ml human recombinant brain-derived neurotrophic factor (BDNF)]; (*c* and *d*) SH-SY5Y cells growing in complete medium without FBS; (*e* and *f*) human glioblastoma-astrocytoma U-87 MG cells. Results are represented as mean±S.E.M. for $n=8$ for all independent experiments. * Statistically significant difference from the control ($p<0.05$).

conditions, we tested the effects of other human recombinant pro-inflammatory cytokines (IL-1β and IL-18) on SH-SY5Y cells. After 24 h treatment with the pro-inflammatory cytokines IL-1β and IL-18 cell viability was significantly increased when compared to control SH-SY5Y cells ($t=3.810$, $p<0.0001$ and $t=5.242$, $p<0.0001$ respectively) without affecting cell density (Fig. 3*a*, *b*). No effects were observed after 72 h treatment either on cell density or in cell viability (Fig. 3*c*, *d*).

A 72 h exposure to IFN-α decreased cell density by inducing early apoptosis

We found that a prolonged (72 h) IFN-α treatment increased the percentage of BrdU positive cells ($t=2.64$; $p=0.015$ *vs.* vehicle-treated cells) suggesting that the reduction in cell density did not depend on an inhibitory effect of cell proliferation (Fig. 4).

We then measured the Bax:Bcl-2 expression ratio. The protein Bax is a member of the Bcl-2 family that promotes apoptosis whereas Bcl-2 is an apoptosis inhibitor. The Bax:Bcl-2 ratio determines the susceptibility of a cell to apoptosis. After a prolonged exposure (72 h) to IFN-α the Bax:Bcl-2 mRNA ratio was increased in treated cells when compared to controls ($t=-4.455$, $p=0.0001$; Fig. 5*b*). In particular, IFN-α exposure significantly decreased the expression of the apoptosis inhibitor Bcl-2 ($t=-5.34$, $p<0.0001$) without affecting the expression of the apoptosis promoter Bax (see later). Moreover, a 72 h exposure to IFN-α significantly decreased the levels of BDNF mRNA compared to vehicle-treated SH-SY5Y cells ($t=-3.012$, $p<0.01$; Fig. 5*d*). After 24 h, we found that the Bax:Bcl-2 mRNA ratio decreased ($t=-4.561$, $p<0.0001$; Fig. 5*a*), and there were no effects on total BDNF mRNA expression (Fig. 5*c*). Consistent with the increased Bax:Bcl-2 mRNA ratio, after 72 h exposure

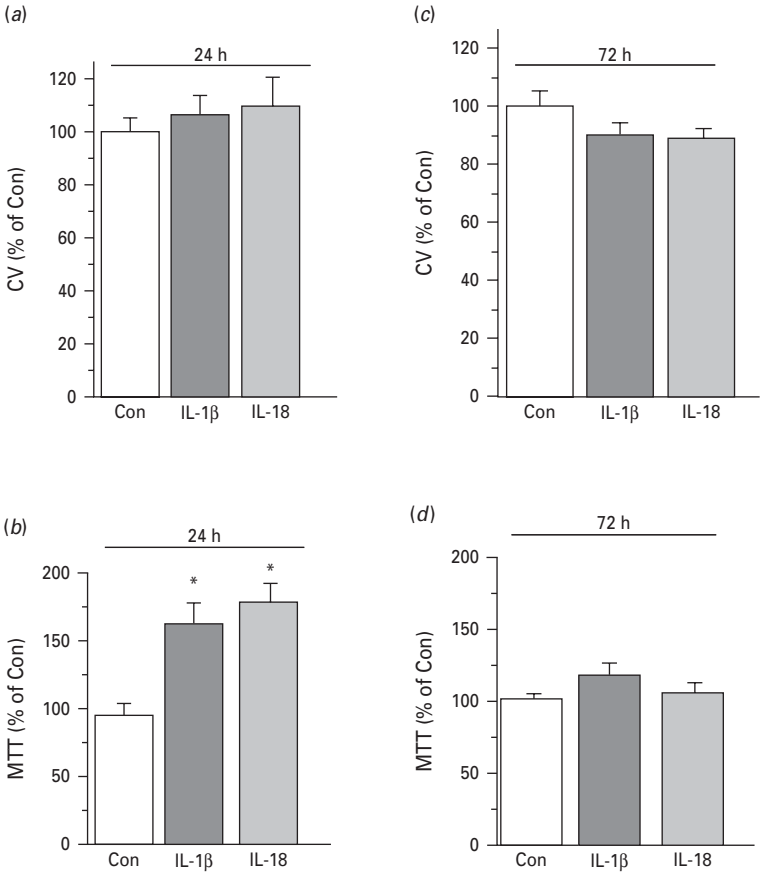

Fig. 3. Cytokine-induced effects against SH-SY5Y cells on cell number [Crystal Violet (CV) staining] and viability [Thiazolyl Blue Tetrazolium Bromide (MTT) assay] after 24 or 72 h. The SH-SY5Y cells at an initial density of 10^4/well were treated with interleukin (IL)-1*β* (10 ng/ml) or IL-18 (100 ng/ml) for 24 (*a* and *b*) or 72 (*c* and *d*) h. (*a*) Acutely, all cytokines tested did not affect cell density compared to vehicle-treated cells (Con), whereas (*c*) no effect was observed after 72 h exposure to IL-1*β* or IL-18 in this *in vitro* model of human neurons. (*b*) Mitochondrial activity was increased 24 h after exposure to all the pro-inflammatory cytokines tested, whereas (*d*) formazan production was not affected after a 72 h exposure to IL-1*β* or IL-18. Results are represented as mean±S.E.M. for $n=8$ for all (three to five) independent experiments. * Statistically significant difference from the control ($p<0.05$).

to IFN-*α* we also found an increased number of cells displaying fragmented nuclei ($t=2.430$, $p=0.002$; Fig. 5*e*) and TUNEL positive cells (Fig. 5*f*).

We found a strong increase in early apoptosis, demonstrated by annexin-V binding (a molecule that binds phosphatidylserine) 72 h after exposure to IFN-*α* treatment ($t=36.73$, $p=0.001$) but not 24 h after (Fig. 5*g*). Moreover, IFN-*α* treatment did not induce significant changes in late apoptosis and necrosis at the two-time points evaluated (24 and 72 h; data not shown).

IFN-α increased the production of H_2O_2 and $mtO_2^{\bullet -}$ after a 72 h exposure in SH-SY5Y

Using a cytofluorimetric approach, we measured simultaneously, at the single-cell level, H_2O_2 content, $mtO_2^{\bullet -}$ and cell physical parameters in control and IFN-*α*-exposed SH-SY5Y cells.

We found a statistically significant increase in the production of both H_2O_2 (+12%, $t=12.31$; $p<0.05$) and $mtO_2^{\bullet -}$ (+230%, $t=3.52$; $p<0.05$) after 72 h exposure to IFN-*α* compared to control SH-SY5Y live cells. No significant effect was observed after 6 or 24 h exposure to IFN-*α* (Fig. 6*a*, *b*).

NAC co-treatment partially prevented IFN-α induced effects

We investigated whether NAC, an antioxidant and mitochondrial modulator with antidepressant properties, could prevent oxidative stress and cytotoxic effects induced by IFN-*α* in SH-SY5Y cells. Cells were

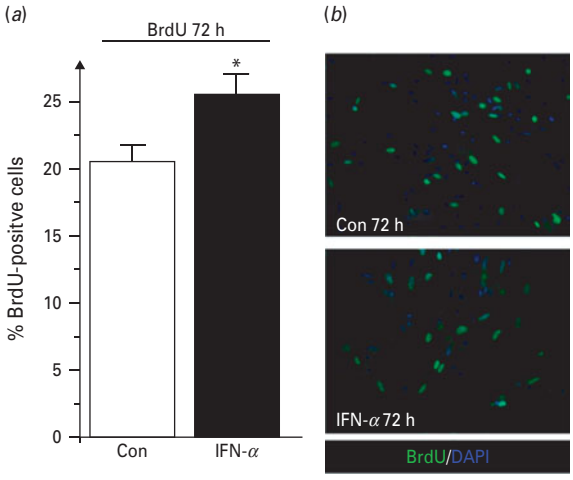

Fig. 4. Paradoxical increase in the proliferation rate in controls (Con) and interferon-α (IFN-α) exposed cells. (*a*) Analysis revealed that 72 h after exposure to IFN-α, the percentage of proliferating 5-bromo-2′-deoxyuridine (BrdU)-positive cells was significantly increased in treated SH-SY5Y cells when compared with vehicle-treated control cells (Con). (*b*) Representative images of BrdU-positive cells (green) and DAPI (blue) staining. Results are represented as mean±S.E.M. * Statistically significant difference from the control ($p<0.05$).

exposed for 72 h to IFN-α (100 ng/ml), NAC (5 mM) or NAC (5 mM) plus IFN-α (100 ng/ml). NAC co-treatment was able to partially prevent IFN-α-induced effects on cell density (Fig. 7*a*): the effects of IFN-α were reduced from −57% to −29%. Indeed, when NAC was co-administered with IFN-α, cell density was significantly different when compared both to control SH-SY5Y cells ($t=-4.45$, $p<0.0001$) and to IFN-α treated cells ($t=5.81$, $p<0.0001$; Fig. 7*a*). NAC also partially counteracted IFN-α-induced effect on cell viability ($t=-3.57$, $p<0.01$ compared to control cells; $t=2.30$, $p<0.05$ compared to IFN-α treated cells; Fig. 7*b*). Again, the effects of IFN-α were reduced from −48% to −29%. In addition, NAC contrasted the effect of IFN-α on cell morphology; for example, cells co-treated with IFN-α plus NAC maintained neurites (Fig. 7*c*). NAC alone did not affect cell density or formazan production.

In term of apoptosis, NAC alone ($t=-5.88$, $p<0.0001$) or in combination with IFN-α ($t=-5.58$, $p<0.0001$) significantly decreased the Bax:Bcl-2 ratio, counteracting the IFN-α-induced effect in increasing the Bax:Bcl-2 ratio (Fig. 7*d*). In particular, exposure to NAC alone increased Bcl-2 mRNA expression ($t=7.14$, $p<0.0001$) and decreased Bax mRNA expression ($t=-2.47$, $p=0.03$; Fig. 7*d*). When cells were exposed to the two compounds together, Bcl-2 expression was significantly higher in treated cells compared to control cells ($t=6.75$, $p<0.0001$) whereas Bax expression was unaffected (Fig. 7*d*). NAC co-administration was also able to prevent IFN-α-induced down-regulation of total BDNF mRNA expression (Fig. 7*e*).

Finally, when cells were exposed to NAC together with IFN-α, NAC was able to prevent ROS production induced by IFN-α (Fig. 8*a*, *b*) as well as an IFN-α-induced effect on early apoptosis (Fig. 9), with NAC alone having no effects.

Discussion

The aim of this paper was to identify some of the molecular and cellular effects of IFN-α on human cells of neuronal origin, paying particular attention to those aspects linked to the potential neurotoxicity of this pro-inflammatory cytokine. We provide for the first time the evidence of a direct toxic effect of IFN-α against the SH-SY5Y cell line, a clone that expresses several properties of human neuronal cells and is largely used as a cellular model to provide mechanistic implication of drug-induced effects on human neurons (Dedoni et al., 2010). In these cells, IFN-α induced apoptosis after 72 h exposure, likely by impairing mitochondrial integrity and activity, recruitment of Bcl-2 family members, and oxidative stress. To strengthen the importance of ROS in such a phenomenon, it is noteworthy that most IFN-α-induced effects on SH-SY5Y cells were counteracted by the antioxidant NAC.

Very few studies have examined the direct effects of IFN-α in neurons, although these cells seem to be a responsive target to the IFN-α-induced central effects (Wang et al., 2008). Type I IFNs (e.g. IFN-α) may have broad-ranging actions in the brain, affecting neuronal differentiation, survival and synaptic plasticity (Ignatowski and Spengler, 2008). Studies in transgenic mice chronically producing IFN-α from astrocytes have highlighted the 'dark side' of the effects of this cytokine in the brain. In fact, deregulated production of IFN-α in the central nervous system induces structural changes, neurodegeneration with loss of neurons (mainly cholinergic neurons), axonal degeneration, impaired neuronal function and disturbed synaptic plasticity (Campbell et al., 1999). However, the molecular mechanisms underlying IFN-α-induced neurodegeneration are still largely unknown.

Consistent with previous studies showing that primary human neuron cultures and neurons produce IFN-α and are responsive to this cytokine (Kawaguchi et al., 1997; Li et al., 2011; Dedoni et al., 2012), we have demonstrated that the SH-SY5Y cell clone expresses

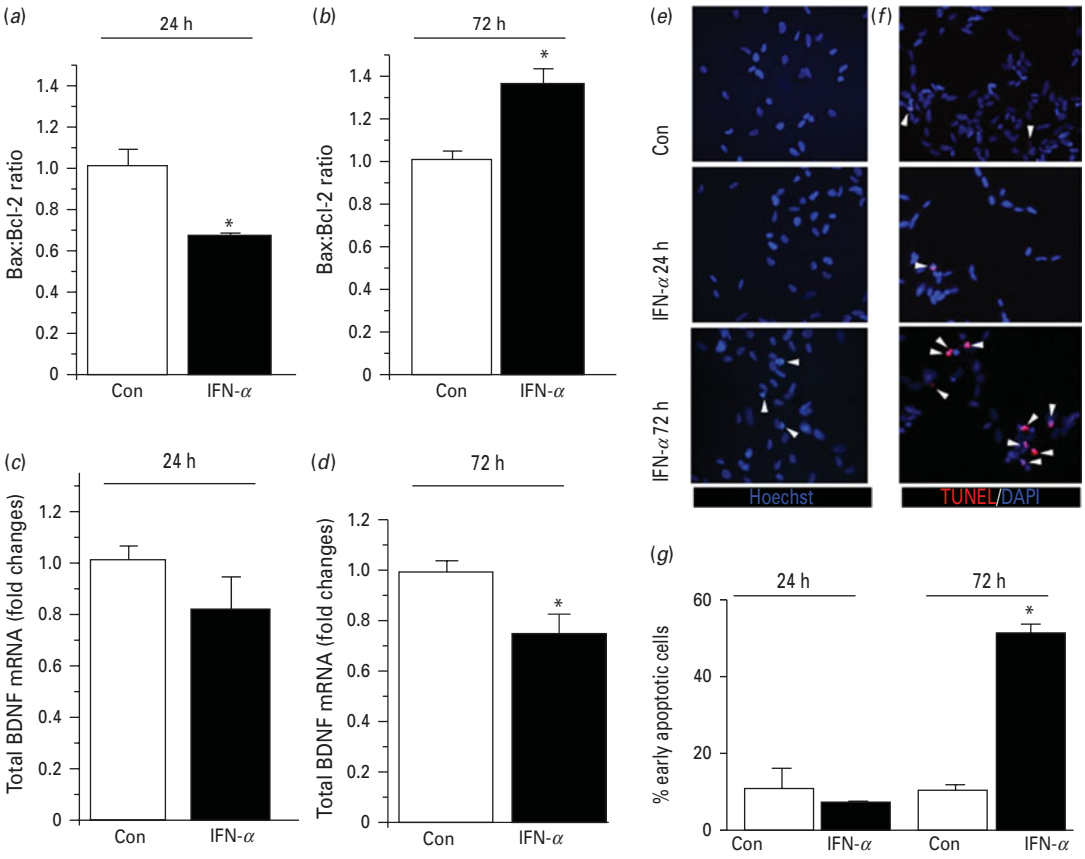

Fig. 5. Interferon-α (IFN-α)-induced apoptosis in SH-SY5Y cells after 72 h exposure. (*a* and *b*) Changes in B-cell CLL/lymphoma 2 (Bcl-2)/Bcl-2-associated X protein (Bax) relative mRNA expression and (*c* and *d*) total brain-derived neurotrophic factor (BDNF) mRNA expression in SH-SY5Y cells after 24 or 72 h treatment. IFN-α treatment was able to alter the Bax:Bcl-2 ratio (<1 after 24 h; >1 after 72 h exposure) and expression levels of the neurotrophin BDNF suggesting that cells died of apoptosis. (*g*) Quantification of early apoptosis in SY-SY5Y cells treated with IFN-α for 24 and 72 h, as revealed by flow cytometry. Results are represented as mean ± S.E.M. for $n=4$ for two independent experiments. * Statistically significant difference from the control ($p<0.05$). Moreover, (*e*) Hoechst staining (blue, marks nuclear chromatin) and (*f*) Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining (red) shows more apoptotic nuclei in IFN-α-exposed cells compared to vehicle-treated cells (Con) at the later time-point tested (72 h). Arrows point to cellular and nuclear morphological features that are indicative of cells undergoing apoptosis.

the IFN-α/IFN-R system. The expression of both the cytokine and its receptor suggests that this pro-inflammatory cytokine may affect cell activity in an autocrine way. The range of the tested doses was decided according with published works and ranged from the dose able to affect glucocorticoid receptor functionality *in vitro* to that comparable to the plasmatic levels observed in IFN-α-treated subjects (Hu et al., 2009; Bekisz et al., 2010; Hayley et al., 2012).

In SH-SY5Y cells, IFN-α causes time- and dose-dependent cytotoxic effects, as demonstrated by using two different tests. The cell number was significantly reduced only after 72 h IFN-α treatment. Neither a 24 nor a 48 h exposure to IFN-α affected the cell density. This may be due to acute adaptive strategies mounted by cells against the immune challenge. Indeed, IFN-α increased the cell viability of SH-SY5Y cells 24 h after exposure. As MTT assay is based on the catalytic activity of enzymes in intact mitochondria, our data suggest that SH-SY5Y may react via mitochondrial protection mechanisms against the immune challenge. This is further supported by a decreased Bax (pro-apoptotic):Bcl-2 (anti-apoptotic) ratio observed at this time (24 h), due to an increased Bcl-2 expression. In the mitochondrion, the balance between the action of pro-death (Bax) or pro-life (Bcl-2) members of the Bcl-2 family determine whether or not pro-apoptotic mediators have to be released from the mitochondria (Yu et al., 2011). IFN-α may have either pro- or anti-apoptotic properties

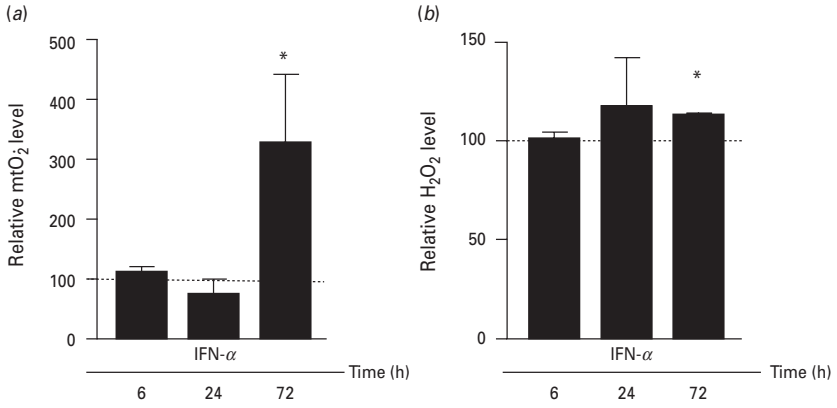

Fig. 6. Effects of interferon-α (IFN-α) on intracellular reactive oxygen species levels. Levels of mitochondrial anion superoxide ($mtO_2^{\bullet -}$) and H_2O_2 in cells treated with IFN-α for 6, 24 or 72 h, as revealed by flow cytometry. A 72 h exposure to IFN-α increased $mtO_2^{\bullet -}$ (*a*) and H_2O_2 (*b*) levels compared to control cells. Data are expressed as percentage change with regard to the control sample, set to 100, and represent the mean ± S.D. of three different experiments. * $p<0.05$ *vs.* control.

depending on the cell types and stimuli (Milner et al., 1995; Rodríguez-Villanueva and McDonnell, 1995; Sangfelt and Strander, 2001). Here we provide evidence that IFN-α-induced effects on apoptosis may depend on the time of exposure and by the ability of the cells to mount an adaptive response to the immune challenge. This may have clinical implications, providing a window for therapeutic intervention to prevent the detrimental effects of a chronic IFN-α treatment. Indeed, at the later time-point (72 h) all IFN-α tested doses reduced the cell density and viability in a concentration-dependent manner.

We also demonstrated that other pro-inflammatory cytokines (IL-1β and IL-18), that are also proposed to mediate neurodegeneration (Ojala et al., 2008; Alboni et al., 2010; Maes et al., 2012), have different effects compared with IFN-α. In fact, all the other cytokines that were able to increase cell viability at 24 h failed to reduce cell density after 72 h exposure. A protective effect for some pro-inflammatory cytokines (e.g. IL-1α and IL-6) in SH-SY5Y cells from oxidative damage has been described, suggesting a neuroprotective role for these cytokines under certain conditions (Bissonnette et al., 2004). Our data show that the cytotoxic effect against the human neuroblastoma SH-SY5Y cell line is specific for IFN-α, and supports the hypothesis that another cell population, like microglia or progenitor cells, may be the target underlying IL-1β and IL-18 mediated neurodegenerative effects in the brain (Sugama et al., 2004; Zunszain et al., 2012a). Indeed, we demonstrated that U-87 MG cells, a human glioblastoma-astrocytoma cell line, do not exhibit a toxic response to IFN-α in the same experimental conditions, showing that only SH-SY5Y cells with a neuronal phenotype exhibit a high toxic response to IFN-α. Obviously, SH-SY5Y cells, although representing a widely accepted *in vitro* model for studies of neurotoxicity, are proliferative cells of tumour origin. However, a peculiarity of these cells is that they could be differentiated by sequential treatment with retinoic acid and BDNF, thus obtaining a homogeneous population of human neuronal mature cells presenting many of the characteristics of primary culture of neurons (Encinas et al., 2000; Agholme et al., 2010). When we tested the cytotoxicity of IFN-α against SH-SY5Y differentiated cells and in serum-deprived condition we obtained similar results as in proliferative cells, suggesting that the IFN-α- induced effect on cell number does not depend on anti-proliferative properties and is maintained in non-proliferative conditions. Indeed, even if IFN-α is known to possess anti-proliferative properties (Bekisz et al., 2010) we evaluated the effects induced by IFN-α exposure in cell proliferation and surprisingly found a paradoxical increase in the SH-SY5Y proliferation rate after 72 h treatment with this cytokine. According to our results, this effect was due to the incorporation of the thymidine analogue (BrdU) in new-born cells *in vitro* and not to DNA repair in post-mitotic cells, because BrdU-labelling was not detected in cells positive for TUNEL (data not shown; Cooper-Kuhn and Kuhn, 2002).

Despite the increased proliferation rate, 72 h exposure to IFN-α resulted in ~50% loss of cellular viability and increased Bax:Bcl-2 ratio as compared to control cells thus suggesting that, at this time, intracellular events triggered by IFN-α are able to impair mitochondrial activity/integrity and may lead to apoptosis. We also found reduced levels of the neurotrophin

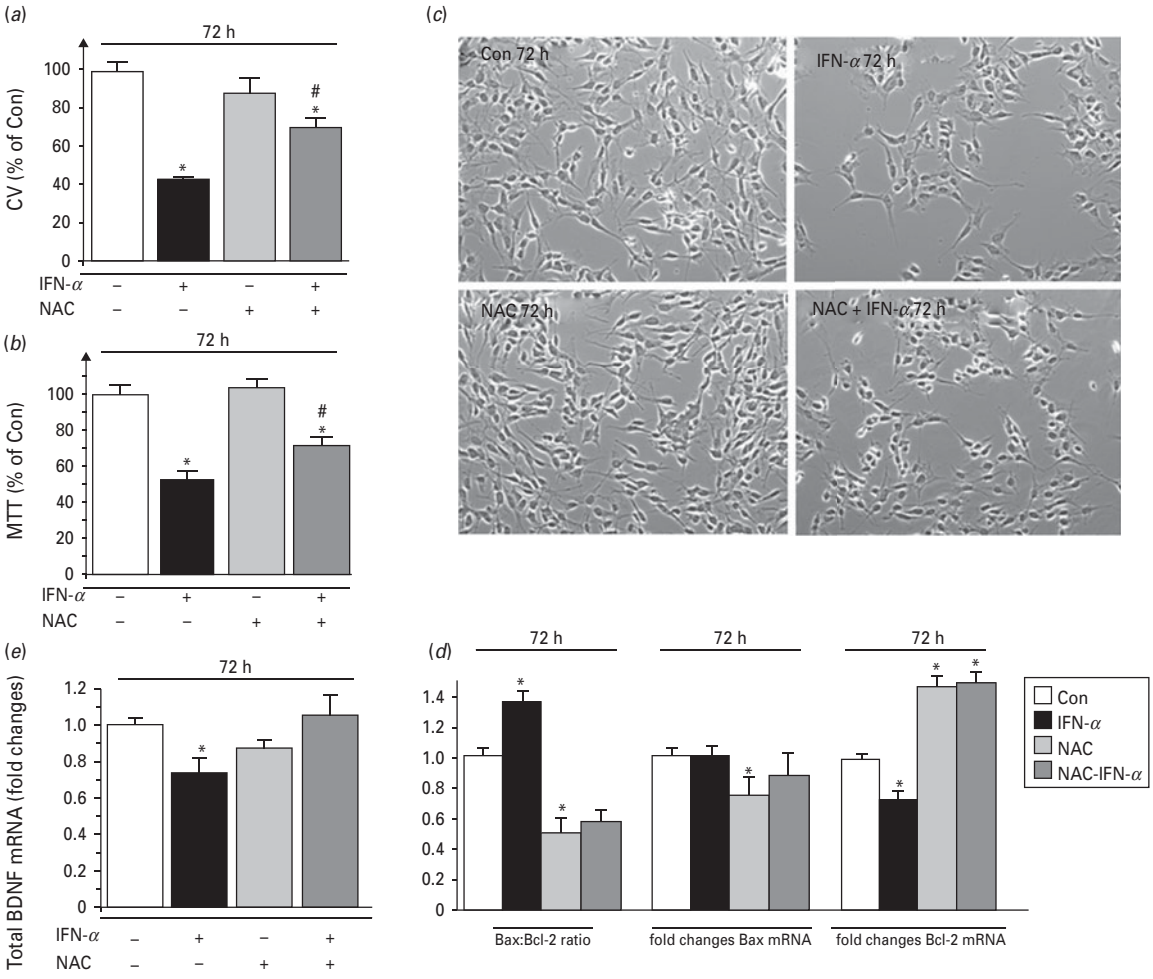

Fig. 7. *N*-acetyl-cysteine (NAC) co-treatment protected SH-SY5Y cells from interferon-α (IFN-α)-induced effects. (*a* and *b*) NAC protected SH-SY5Y cells against cytotoxicity induced by 72 h exposure to IFN-α. (*a*) Cell density and (*b*) formazan production were significantly higher in SH-SY5Y cells exposed to NAC+IFN-α compared to cells treated for 72 h with IFN-α. Results are represented as mean±S.E.M. for $n=8$ for two independent experiments. * Statistically significant difference from the control ($p<0.05$). (*c*) Phase-contrast photomicrographs (×20 objective) of representative SH-SY5Y cells showing morphological changes 72 h after treatments with IFN-α, NAC or NAC+IFN-α. (*d*) Relative abundance of B-cell CLL/lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) mRNA expression and Bax:Bcl-2 mRNA expression in SH-SY5Y cells exposed to IFN-α, NAC or NAC+IFN-α for 72 h. NAC co-administration with IFN-α increased the expression of the anti-apoptotic *bcl-2* gene thus resulting in a reduced Bax:Bcl-2 ratio. (*e*) Total brain-derived neurotrophic factor (BDNF) mRNA expression in SH-SY5Y cells exposed to IFN-α, NAC or NAC+IFN-α for 72 h. NAC alone did not affect BDNF expression but was able to prevent IFN-α- induced effect on BDNF gene expression. The relative abundance of each transcript was normalized with ubiquitin C and an average of vehicle-treated cells was used as calibrator. Results are represented as mean±S.E.M. for $n=4$ for two independent experiments. * Statistically significant difference from the control ($p<0.05$).

BDNF expression after 72 h IFN-α treatment. Deregulation of BDNF signalling is involved in the pathogenesis of neurodegenerative and neuropsychiatric disorders (Alboni et al., 2011a; Zhang et al., 2012). Moreover, in HCV patients receiving IFN-α treatment, serum levels of BDNF are inversely associated with depressive symptoms, suggesting that IFN-α-induced depression may be partially explained by alteration in the neuroprotective capacity (Kenis et al., 2011). It was recently reported that prolonged exposure to type I IFNs curtails BDNF-induced signalling, cell survival and neurite outgrowth in SH-SY5Y cells (Dedoni et al., 2012). Reduced neurotrophic support may contribute to mediated IFN-α-induced toxicity against human neuron like cells undergoing apoptosis (Franklin, 2011). It has been reported that,

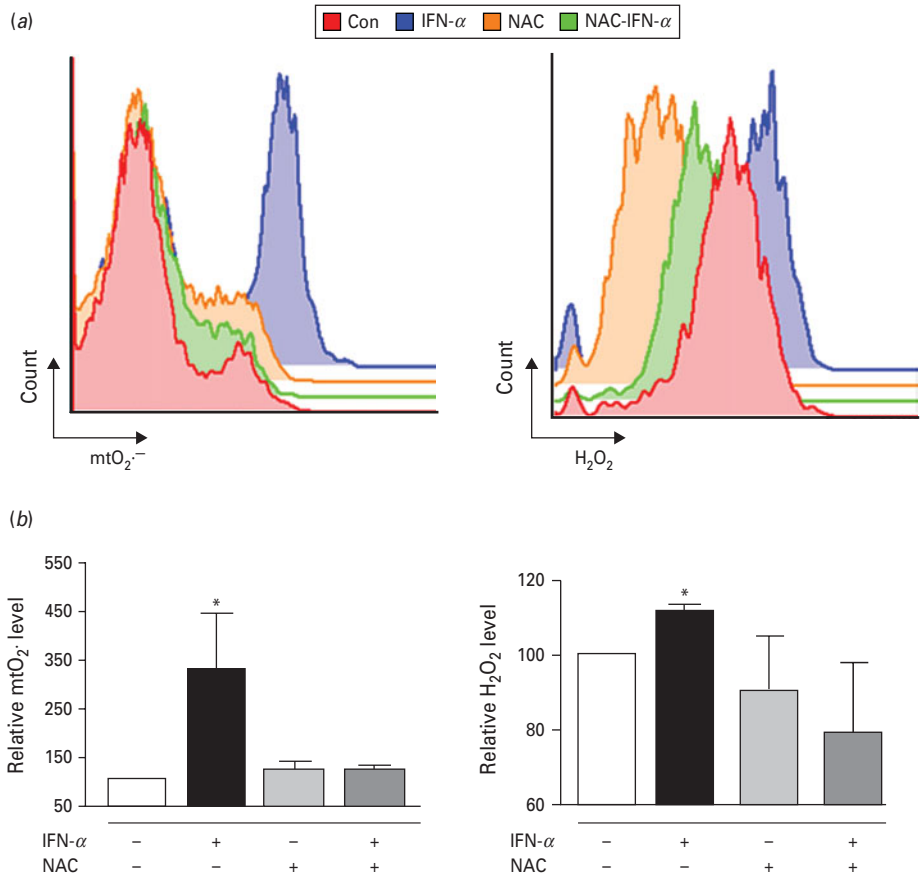

Fig. 8. Effects of interferon-α (IFN-α) and *N*-acetyl-cysteine (NAC) co-treatment on intracellular reactive oxygen species levels. (*a*) Representative histograms showing the effects of IFN-α, NAC and the co-treatment on the intracellular fluorescence of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFH-DA; right panel) and of MitoSOX Red superoxide indicator (left panel). (*b*) Levels of H_2O_2 and mitochondrial anion superoxide ($mtO_2^{\bullet -}$) in cells treated with IFN-α, NAC, and the combination of both, for 72 h, as revealed by flow cytometry. Data are expressed as percentage change with respect to the control sample, set to 100, and represent mean±S.D. of three different experiments. * $p<0.05$ *vs.* control.

in neurons, neurotrophins (e.g. BDNF) exert neuroprotective effects by affecting mitochondrial function (Markham et al., 2012), regulating Bcl-2 family members (Pérez-Navarro et al., 2005) and through activation of the antioxidant pathway. Therefore, it is possible that IFN-α and BDNF regulate similar mechanisms but in opposite directions, or that the effects of IFN-α on mitochondrial function and apoptosis are mediated in part by the decrease of BDNF activity.

By polychromatic flow cytometry we evaluated IFN-α-induced effect on early apoptosis, late apoptosis or necrosis. We found increased early apoptosis at the later time-point, suggesting that IFN-α induced neuronal death through an intrinsic pathway very likely evoked by the binding of the cytokine to its receptor, but also that other events triggered by the formation of the IFN-α/IFN-R are eventually responsible for the reduced cell density observed after 72 h IFN-α exposure. It has been reported that IFN-α effects on neurons are mediated in part by the production of reactive oxygen intermediates (Hori et al., 1998). Indeed, among the stimuli that can induce cells to undergo apoptotic death, one of the most reproducible is mild oxidative stress (Slater et al., 1995). It has long been known that increased levels of ROS such as H_2O_2 and the superoxide anion $O_2^{\bullet -}$ occur in neurons undergoing apoptotic death (Greenlund et al., 1995). Elevated ROS production is now recognized as a necessary event of apoptosis, by generating a cellular pro-oxidative state (Franklin, 2011). Elevated production of ROS occurring in neurons has been consistently linked to the origin and pathology of several diseases, including neurodegenerative and psychiatric disorders as well as in normal ageing (Finch, 2007;

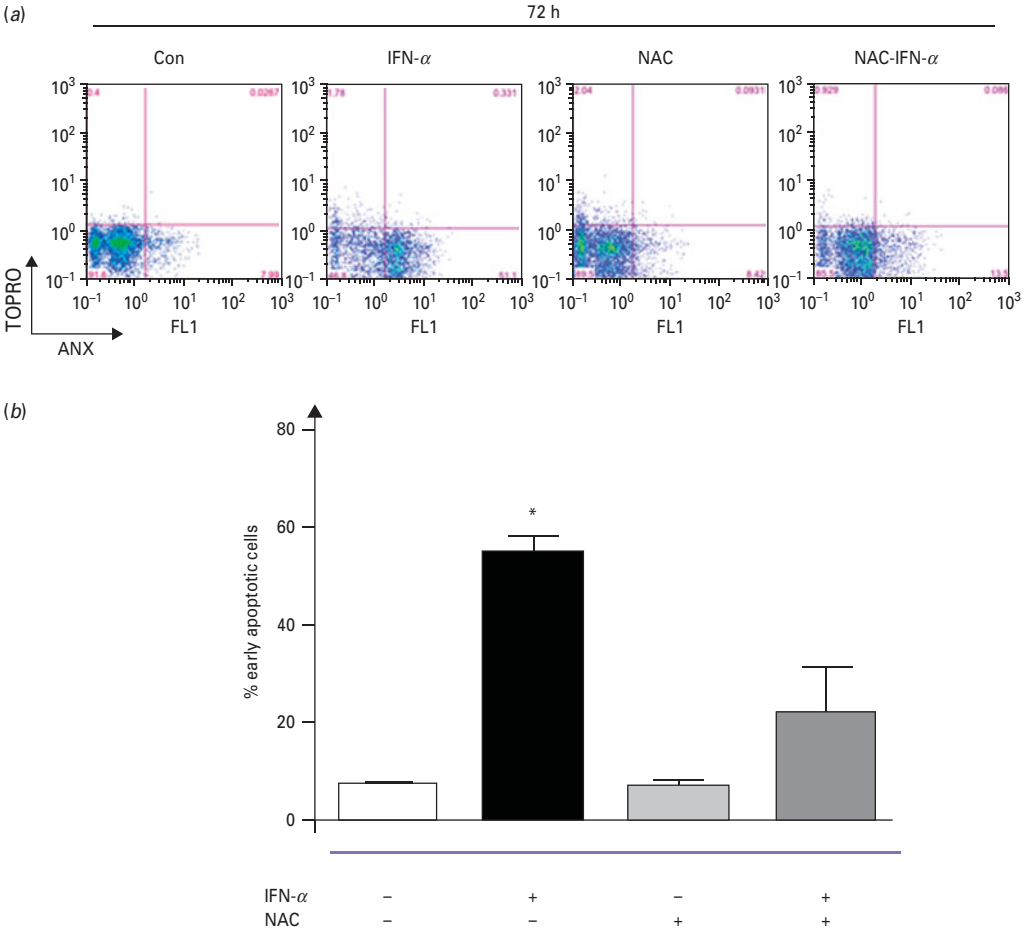

Fig. 9. Effects of interferon-α (IFN-α) and *N*-acetyl-cysteine (NAC) co-treatment on apoptosis. (*a*) Representative dot plots combining annexin-V (ANX) and TO-PRO-3 iodide (TOPRO) staining in SH-SY5Y cells treated with IFN-α, NAC, and the combination of both, for 72 h. ANX/TOPRO assay allows the identification of four cell populations: ANX−/TOPRO− (viable) cells; ANX+/TOPRO− (early apoptotic) cells; ANX+/TOPRO+ (late apoptotic) cells; ANX−/TOPRO+ (necrotic) cells. (*b*) Quantification of early apoptotic cells after treatment with IFN-α, NAC, and the combination of both for 72 h, as revealed by flow cytometry. Data represent mean±S.D. of three different experiments. * $p<0.05$ *vs.* control.

Halliwell and Gutteridge, 2007; Leonard and Maes, 2012). We found a significant increase in production of H_2O_2 and $mtO_2^{\bullet -}$ after 72 h exposure to IFN-α, suggesting that impaired pro-oxidant state contributes to undergoing apoptosis in SH-SY5Y cells. According with this hypothesis, we demonstrated that co-treatment with the antioxidant membrane permeable NAC was able to counter many of the detrimental IFN-α-induced effects in our *in vitro* model of human neurons. In these cells, NAC co-administration partially counteracted IFN-α-induced effects on cell density, mitochondrial metabolism and cell morphology, while it blocked the effects of ROS production and apoptosis, as already described in other models (Cossarizza et al., 1995). Moreover, after 72 h, NAC alone or in combination with IFN-α increased Bcl-2 expression and decreased the Bax:Bcl-2 ratio towards an increased cell resistance. Inhibition of Bax induction following an oxidative stress by NAC was already demonstrated in these cells (Watcharasit et al., 2010). Finally, whereas NAC alone did not affect BDNF expression after 72 h exposure, NAC co-administration prevented the reduction in BDNF expression observed after IFN-α treatment, thus contributing to maintaining the neurotrophic support and further strengthening the role for oxidative stress and inflammation in regulating BDNF levels. In cells, NAC is rapidly metabolized to intracellular glutathione (GSH). GSH is the primary endogenous antioxidant in the brain with an important role in the detoxification and in the prevention from damage due to ROS. Therefore, an NAC protective effect from IFN-α-induced toxicity

in SH-SY5Y cells may be in part due to activation of the GSH redox-cycling pathway, ROS suppression, prevention of Bax induction and apoptosis regulation. NAC has been shown to have effects on antioxidant status, glutamate transmission, inflammation and neurogenesis (Dean et al., 2012). It has been recently demonstrated that NAC may prevent memory deficits in models of neurodegenerative conditions or ageing by counteracting oxidative damage (Smaga et al., 2012). Moreover, NAC could prevent depressive-like behaviour induced by exposure to negative challenges by counteracting stress-induced oxidative damage in the prefrontal cortex, the hippocampus and the amygdala, brain areas most involved in depression (Arent et al., 2012).

The use of NAC, together with other mitochondrial modulators has been suggested for the management of bipolar disorder (Nierenberg et al., 2013). Recent clinical studies have demonstrated a robust effect of NAC in treating depressive symptoms (Magalhães et al., 2011; Berk et al., 2012).

In conclusion, we demonstrate that neurons are a direct target of IFN-α-induced neurotoxic effects. Of course we can only speculate that these *in vitro* observed molecular events induced by IFN-α exposure may be in part responsible for IFN-α-induced neurodegeneration and/or psychiatric side-effects. Nevertheless, it is interesting to propose that NAC, a safe drug also effective in treating liver failure, could be used to prevent IFN-α-induced effects. Although evidence about the ability of NAC to penetrate the BBB are conflicting (McLellan et al., 1995; Neuwelt et al., 2001), peripheral administration of NAC prevents GSH depletion in the brain (Berk et al., 2008a, b) thus exerting a protective effect against pro-oxidative state induced by different challenges (Scapagnini et al., 2012). Further clinical studies may support a protective effect for NAC in preventing severe side-effects of IFN-α.

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Statement of Interest

None.

Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145713000266

References

Agholme L, Lindström T, Kågedal K, Marcusson J, Hallbeck M (2010) An *in vitro* model for neuroscience: differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons. J Alzheimers Dis 20:1069–1082.

Alboni S, Cervia D, Sugama S, Conti B (2010) Interleukin 18 in the CNS. J Neuroinflammation 7:9.

Alboni S, Montanari C, Benatti C, Blom JM, Simone ML, Brunello N, Caggia F, Guidotti G, Marcondes MC, Sanchez-Alavez M, Conti B, Tascedda F (2011a) Constitutive and LPS-regulated expression of interleukin-18 receptor beta variants in the mouse brain. Brain Behav Immun 25:483–493.

Alboni S, Tascedda F, Corsini D, Benatti C, Caggia F, Capone G, Barden N, Blom JM, Brunello N (2011b) Stress induces altered CRE/CREB pathway activity and BDNF expression in the hippocampus of glucocorticoid receptor-impaired mice. Neuropharmacology 60:1337–1346.

Arent CO, Réus GZ, Abelaira HM, Ribeiro KF, Steckert AV, Mina F, Dal-Pizzol F, Quevedo J (2012) Synergist effects of *N*-acetylcysteine and deferoxamine treatment on behavioral and oxidative parameters induced by chronic mild stress in rats. Neurochem Int 61:1072–1080.

Averhoff FM, Glass N, Holtzman D (2012) Global burden of hepatitis C: considerations for healthcare providers in the United States. Clin Infect Dis 55 (Suppl. 1):S10–S15.

Baraldi S, Hepgul N, Mondelli V, Pariante CM (2012) Symptomatic treatment of interferon-α-induced depression in hepatitis C: a systematic review. J Clin Psychopharmacol 32:531–543.

Bekisz J, Baron S, Balinsky C, Morrow A, Zoon KC (2010) Antiproliferative properties of type I and type II interferon. Pharmaceuticals (Basel) 3:994–1015.

Berk M, Copolov D, Dean O, Lu K, Jeavons S, Schapkaitz I, Anderson-Hunt M, Judd F, Katz F, Katz P, Ording-Jespersen S, Little J, Conus P, Cuenod M, Do KQ, Bush AI (2008a) *N*-acetyl-cysteine as a glutathione precursor for schizophrenia – a double-blind, randomized, placebo-controlled trial. Biol Psychiatry 64:361–368.

Berk M, Copolov DL, Dean O, Lu K, Jeavons S, Schapkaitz I, Anderson-Hunt M, Bush AI (2008b) *N*-acetyl-cysteine for depressive symptoms in bipolar disorder – a double-blind randomized placebo-controlled trial. Biol Psychiatry 64:468–465.

Berk M, Dean OM, Cotton SM, Gama CS, Kapczinski F, Fernandes B, Kohlmann K, Jeavons S, Hewitt K, Moss K, Allwang C, Schapkaitz I, Cobb H, Bush AI, Dodd S, Malhi GS (2012) Maintenance *N*-acetyl-cysteine treatment for bipolar disorder: a double-blind randomized placebo

controlled trial. BMC Med. Advance online publication. Retrieved 4 Aug 2012. doi: 10.1186/1741-7015-10-91.

Bissonnette CJ, Klegeris A, McGeer PL, McGeer EG (2004) Interleukin 1 alpha and interleukin 6 protect human neuronal SH-SY5Y cells from oxidative damage. Neurosci Lett 361:40–43.

Campbell IL, Krucker T, Steffensen S, Akwa Y, Powell HC, Lane T, Carr DJ, Gold LH, Henriksen SJ, Siggins GR (1999) Structural and functional neuropathology in transgenic mice with CNS expression of IFN-alpha. Brain Res 835:46–61.

Cooper-Kuhn CM, Kuhn HG (2002) Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. Brain Res Dev Brain Res 134:13–21.

Cossarizza A, Franceschi C, Monti D, Salvioli S, Bellesia E, Rivabene R, Biondo L, Rainaldi G, Tinari A, Malorni W (1995) Protective effect of *N*-acetylcysteine in tumor necrosis factor-alpha-induced apoptosis in U937 cells: the role of mitochondria. Exp Cell Res 220:232–240.

Cossarizza A, Ferraresi R, Troiano L, Roat E, Gibellini L, Bertoncelli L, Nasi M, Pinti M (2009) Simultaneous analysis of reactive oxygen species and reduced glutathione content in living cells by polychromatic flow cytometry. Nat Protoc 4:1790–1797.

Dean OM, Bush AI, Copolov DL, Kohlmann K, Jeavons S, Schapkaitz I, Anderson-Hunt M, Berk M (2012) Effects of *N*-acetyl-cysteine on cognitive function in bipolar disorder. Psychiatry Clin Neurosci 66:514–517.

Dedoni S, Olianas MC, Onali P (2010) Interferon-β induces apoptosis in human SH-SY5Y neuroblastoma cells through activation of JAK-STAT signaling and down-regulation of PI3K/Akt pathway. J Neurochem 115:1421–1433.

Dedoni S, Olianas MC, Ingianni A, Onali P (2012) Type I interferons impair BDNF-induced cell signaling and neurotrophic activity in differentiated human SH-SY5Y neuroblastoma cells and mouse primary cortical neurons. J Neurochem 122:58–71.

Donnici L, Tiraboschi E, Tardito D, Musazzi L, Racagni G, Popoli M (2008) Time-dependent biphasic modulation of human BDNF by antidepressants in neuroblastoma cells. BMC Neurosci 5:9–61.

Emanuelsson I, Norlin M (2012) Protective effects of 27- and 24-hydroxycholesterol against staurosporine-induced cell death in undifferentiated neuroblastoma SH-SY5Y cells. Neurosci Lett 525:44–48.

Encinas M, Iglesias M, Liu Y, Wang H, Muhaisen A, Ceña V, Gallego C, Comella JX (2000) Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells. J Neurochem 75:991–1003.

Felger JC, Alagbe O, Hu F, Mook D, Freeman AA, Sanchez MM, Kalin NH, Ratti E, Nemeroff CB, Miller AH (2007) Effects of interferon-alpha on rhesus monkeys: a nonhuman primate model of cytokine-induced depression. Biol Psychiatry 62:1324–1333.

Finch CE (2007) The biology of human longevity: inflammation, nutrition, and aging in the evolution of lifespans. San Diego: Elsevier.

Fioravanti J, Medina-Echeverz J, Ardaiz N, Gomar C, Parra-Guillén ZP, Prieto J, Berraondo P (2012) The fusion protein of IFN-α and apolipoprotein A-I crosses the blood–brain barrier by a saturable transport mechanism. J Immunol 188:3988–3992.

Franklin JL (2011) Redox regulation of the intrinsic pathway in neuronal apoptosis. Antioxid Redox Signal 14:1437–1448.

Greenlund LJ, Deckwerth TL, Johnson EM Jr (1995) Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death. Neuron 14:303–315.

Halliwell B, Gutteridge JMC (2007) Free radicals in biology and medicine, 4th edn. Oxford: Oxford University Press.

Hayley S, Scharf J, Anisman H (2012) Central administration of murine interferon-α induces depressive-like behavioral, brain cytokine and neurochemical alterations in mice: a mini-review and original experiments. Brain Behav Immun Advance online publication. Retrieved 4 Aug 2012. doi: 10.1016/j.bbi.2012.07.023

Hepgul N, Kodate N, Anderson JE, Henderson M, Ranjith G, Hotopf M, Pariante CM (2012) Understanding clinical risk decision making regarding development of depression during interferon-alpha treatment for hepatitis-C: a - qualitative interview study. Int J Nurs Stud 49:1480–1488.

Hori T, Katafuchi T, Take S, Shimizu N (1998) Neuroimmunomodulatory actions of hypothalamic interferon-alpha. Neuroimmunomodulation 5:172–177.

Hu F, Pace TW, Miller AH (2009) Interferon-alpha inhibits glucocorticoid receptor-mediated gene transcription via STAT5 activation in mouse HT22 cells. Brain Behav Immun 23:455–463.

Ignatowski TA and Spengler RN (2008) Cytokines and the brain (neuroimmune biology). Amsterdam: Elsevier.

Indraccolo S, Pfeffer U, Minuzzo S, Esposito G, Roni V, Mandruzzato S, Ferrari N, Anfosso L, Dell'Eva R, Noonan DM, Chieco-Bianchi L, Albini A, Amadori A (2007) Identification of genes selectively regulated by IFNs in endothelial cells. J Immunol 178:1122–1135.

Kawaguchi N, Yamada T, Yoshiyama Y (1997) Expression of interferon-alpha mRNA in human brain tissues. No To Shinkei 49:69–73.

Kenis G, Prickaerts J, van Os J, Koek GH, Robaeys G, Steinbusch HW, Wichers M (2011) Depressive symptoms following interferon-α therapy: mediated by immune-induced reductions in brain-derived neurotrophic factor? Int J Neuropsychopharmacol 14:2472–2453.

Leonard B, Maes M (2012) Mechanistic explanations how cell-mediated immune activation, inflammation and oxidative and nitrosative stress pathways and their sequels and concomitants play a role in the pathophysiology of unipolar depression. Neurosci Biobehav 36:764–785.

Leutscher PD, Lagging M, Buhl MR, Pedersen C, Norkrans G, Langeland N, Mørch K, Färkkilä M, Hjerrild S, Hellstrand

K, Bech P; NORDynamIC Group (2010) Evaluation of depression as a risk factor for treatment failure in chronic hepatitis C. Hepatology 52:430–435.

Li J, Hu S, Zhou L, Ye L, Wang X, Ho J, Ho W (2011) Interferon lambda inhibits herpes simplex virus type I infection of human astrocytes and neurons. Glia 59:58–67.

McLellan LI, Lewis AD, Hall DJ, Ansell JD, Wolf CR (1995) Uptake and distribution of *N*-acetylcysteine in mice: tissue-specific effects on glutathione concentrations. Carcinogenesis 16:2099–2106.

Maddock C, Landau S, Barry K, Maulayah P, Hotopf M, Cleare AJ, Norris S, Pariante CM (2005) Psychopathological symptoms during interferon-alpha and ribavirin treatment: effects on virologic response. Mol Psychiatry 10:332–333.

Maes M, Song C, Yirmiya R (2012) Targeting IL-1 in depression. Expert Opin Ther Targets 16:1097–1112.

Magalhães PV, Dean OM, Bush AI, Copolov DL, Malhi GS, Kohlmann K, Jeavons S, Schapkaitz I, Anderson-Hunt M, Berk M (2011) *N*-acetylcysteine for major depressive episodes in bipolar disorder. Rev Bras Psiquiatr 33:374–378.

Markham A, Cameron I, Bains R, Franklin P, Kiss JP, Schwendimann L, Gressens P, Spedding M (2012) Brain-derived neurotrophic factor-mediated effects on mitochondrial respiratory coupling and neuroprotection share the same molecular signalling pathways. Eur J Neurosci 35:366–374.

Milner AE, Grand RJ, Gregory CD (1995) Effects of interferon-alpha on human B cells: repression of apoptosis and prevention of cell growth are independent responses of Burkitt lymphoma lines. Int J Cancer 61:348–354.

Neuwelt EA, Pagel MA, Hasler BP, Deloughery TG, Muldoon LL (2001) Therapeutic efficacy of aortic administration of *N*-acetylcysteine as a chemoprotectant against bone marrow toxicity after intracarotid administration of alkylators, with or without glutathione depletion in a rat model. Cancer Res 61:7868–7874.

Nierenberg AA, Kansky C, Brennan BP, Shelton RC, Perlis R, Iosifescu DV (2013) Mitochondrial modulators for bipolar disorder: a pathophysiologically informed paradigm for new drug development. Aust N Z J Psychiatry 47:26–42.

Ojala JO, Sutinen EM, Salminen A, Pirttilä T (2008) Interleukin-18 increases expression of kinases involved in tau phosphorylation in SH-SY5Y neuroblastoma cells. J Neuroimmunol 205:86–93.

Pérez-Navarro E, Gavaldà N, Gratacòs E, Alberch J (2005) Brain-derived neurotrophic factor prevents changes in Bcl-2 family members and caspase-3 activation induced by excitotoxicity in the striatum. J Neurochem 92:678–691.

Pirkmajer S, Chibalin AV (2011) Serum starvation: caveat emptor. Am J Physiol Cell Physiol 30:C272–C279.

Rodríguez-Villanueva J, McDonnell TJ (1995) Induction of apoptotic cell death in non-melanoma skin cancer by interferon-alpha. Int J Cancer 61:110–114.

Sangfelt O, Strander H (2001) Apoptosis and cell growth inhibition as antitumor effector functions of interferons. Med Oncol 18:3–14.

Scapagnini G, Davinelli S, Drago F, De Lorenzo A, Oriani G (2012) Antioxidants as antidepressants: fact or fiction? CNS Drugs 26:477–490.

Schaefer M, Capuron L, Friebe A, Diez-Quevedo C, Robaeys G, Neri S, Foster GR, Kautz A, Forton D, Pariante CM (2012) Hepatitis C infection, antiviral treatment and mental health: a European expert consensus statement. J Hepatol 57:1379–1390.

Slater AF, Stefan C, Nobel I, van den Dobbelsteen DJ, Orrenius S (1995) Signalling mechanisms and oxidative stress in apoptosis. Toxicol Lett 82–83:149–153.

Smaga I, Pomierny B, Krzyżanowska W, Pomierny-Chamioło L, Miszkiel J, Niedzielska E, Ogórka A, Filip M (2012) *N*-acetylcysteine possesses antidepressant-like activity through reduction of oxidative stress: behavioral and biochemical analyses in rats. Prog Neuropsychopharmacol Biol Psychiatry 39:280–287.

Sugama S, Wirz SA, Barr AM, Conti B, Bartfai T, Shibasaki T (2004) Interleukin-18 null mice show diminished microglial activation and reduced dopaminergic neuron loss following acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment. Neuroscience 128:451–458.

Sutinen EM, Pirttilä T, Anderson G, Salminen A, Ojala JO (2012) Pro-inflammatory interleukin-18 increases Alzheimer's disease-associated amyloid-β production in human neuron-like cells. J Neuroinflammation 16:9–199.

Sy T, Jamal MM (2006) Epidemiology of hepatitis C virus (HCV) infection. Int J Med Sci 3:41–46.

Troiano L, Ferraresi R, Lugli E, Nemes E, Roat E, Nasi M, Pinti M, Cossarizza A (2007) Multiparametric analysis of cells with different mitochondrial membrane potential during apoptosis by polychromatic flow cytometry. Nat Protoc 2:2719–2727.

Vitkovic L, Konsman JP, Bockaert J, Dantzer R, Homburger V, Jacque C (2000) Cytokine signals propagate through the brain. Mol Psychiatry 5:604–615.

Wang J, Campbell IL, Zhang H (2008) Systemic interferon-alpha regulates interferon-stimulated genes in the central nervous system. Mol Psychiatry 13:293–301.

Wartelle-Bladou C, Le Folgoc G, Bourlière M, Lecomte L (2012) Hepatitis C therapy in non-genotype 1 patients: the near future. J Viral Hepat 19:525–536.

Watcharasit P, Suntararuks S, Visitnonthachai D, Thiantanawat A, Satayavivad J (2010) Acrylonitrile induced apoptosis via oxidative stress in neuroblastoma SH-SY5Y cell. J Appl Toxicol 30:649–655.

Xie HR, Hu LS, Li GY (2010) SH-SY5Y human neuroblastoma cell line: *in vitro* cell model of dopaminergic neurons in Parkinson's disease. Chin Med J (Engl) 123:1086–1092.

Yu J, Ye J, Liu X, Han Y, Wang C (2011) Protective effect of L-carnitine against H(2)O(2)-induced neurotoxicity in neuroblastoma (SH-SY5Y) cells. Neurol Res 33:708–716.

Zhang F, Kang Z, Li W, Xiao Z, Zhou X (2012) Roles of brain-derived neurotrophic factor/tropomyosin-related

kinase B (BDNF/TrkB) signalling in Alzheimer's disease. J Clin Neurosci 19:946–949.

Zunszain PA, Anacker C, Cattaneo A, Choudhury S, Musaelyan K, Myint AM, Thuret S, Price J, Pariante CM (2012a) Interleukin-1β: a new regulator of the kynurenine pathway affecting human hippocampal neurogenesis. Neuropsychopharmacology 37:939–949.

Zunszain PA, Hepgul N, Pariante CM (2012b) Inflammation and depression. Curr Top Behav Neurosci. Advance online publication. Retrieved 3 May 2012. doi:10.1007/7854_2012_211