# Impact of interferon gamma on murine- and human embryonic stem cell-derived neural cell populations

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**Introductory remarks:**

Parts of this work, carried out at the Department of Neurology, University Hospital Düsseldorf and partially at the Hertie-Institute for Clinical Brain Research in Tübingen have been published, or have been displayed in manuscripts that are in review. This PhD thesis combines three publications, which are associated by their scientific content. A list of all publications is given at the end of this PhD thesis.

**Abstract**

Interferon gamma (IFNγ) is a proinflammatory cytokine that is secreted by various cell types during inflammation, mostly by cytotoxic $CD8^+$ T-cells and natural killer cells [1] but also by astrocytes, fibroblasts and endothelial cells [2,3,4]. Inflammation is a main mediator of neuronal and glial cell death in various neurological diseases such as stroke, acute brain traumata, HIV-mediated dementia, multiple sclerosis, amyotrophic lateral sclerosis and others [5,6]. Neural stem cell therapy is aimed at cell and tissue replacement under pathological conditions, at stimulating dormant endogenous stem cell niches or at using transplanted cells as transducers for stimulating factors and expression of gene products that have a therapeutic effect (ex vivo gene therapy) [7]. Since it is known that the amount of IFNγ increases under pathological conditions and neural stem cell therapy is aimed at cell transplantation into diseased brain areas, the impact of IFNγ on neural stem cells needed to be explored. For this reason, we started our investigations at the level of murine neural stem cells and characterized the effects of IFNγ. Then we further clarified the underlying molecular pathways involved in the reactions of murine neural stem cells to IFNγ. After investigating the genotypic and phenotypic reactions of murine cells to IFNγ, we examined IFNγ-related reactions of human neural stem cells, that were derived from human embryonic stem cells. By using a model system on a human genetic background, we hope that the predictability concerning the human diseased situation is more appropriate.

**Zusammenfassung**

Interferon gamma (IFNγ) ist ein proinflammatorisches Zytokin, das hauptsächlich von zytotoxischen $CD8^+$ T-Zellen [1] und natürlichen Killerzellen, aber auch von Astrozyten, Fibroblasten und Endothelzellen während entzündlicher Prozesse sezerniert wird [2,3,4]. Während einer Vielzahl neurologischer Erkrankungen wie dem Schlaganfall, Schädel-Hirn-Traumata, der HIV-assoziierten Demenz, der Multiplen Sklerose und der amyothropen Lateralsklerose wird neuronaler und glialer Zelltod durch eben solche entzündlichen Prozesse ausgelöst und führt zu teils schweren funktionellen Defiziten [5,6].

Mögliche Ziele einer neuralen Stammzelltherapie sind, zerstörte oder erkrankte Zellareale durch Transplantation zu ersetzen, inaktive Stammzellnischen im adulten Gehirn zu aktivieren oder stimulierende Substanzen und die Expression von therapeutisch wirksamen Genprodukten durch transplantierte Zellen freizusetzen [7]. Da IFNγ in den erkrankten Gehirnarealen in hohem Maße vorhanden ist, ist es wichtig, den Effekt von IFNγ auf neurale Stammzellen zu untersuchen. Aus diesem Grunde starteten wir unsere Untersuchungsreihe zur Charakterisierung der Effekte von IFNγ auf neurale Stammzellen. Zu Beginn verwendeten wir murine neurale Stammzellen und untersuchten die phänotypischen und genotypischen Effekte von IFNγ auf diesen Zelltyp. Danach führten wir weitere molekularbiologische Untersuchungen durch, um die beobachteten Effekte aufzuklären. Nach Abschluss der Untersuchungen auf muriner Ebene, untersuchten wir den Einfluss von IFNγ auf humane neurale Stammzellen, die aus humanen embryonalen Stammzellen hergestellt wurden, da dieses Zellkultursystem den Verhältnissen, die in humanen Krankheiten vorkommen, ähnlicher ist als eines auf der Basis muriner Zellen.

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# 1. Introduction

## 1.1 Interferon gamma

Interferon gamma (IFNγ) is a protein that belongs to the family of interferons. This family contains Interferon alpha (IFNα) and Interferon beta (IFNβ) next to IFNγ. IFNα and IFNβ are produced and secreted by cells as a reaction against viral infections. Both cytokines bind to the same receptor in the cell membrane and are referred as Type I interferons. The structure of IFNγ on genetic and on protein level is different to IFNα and IFNβ. It is secreted by other cell types, binds to a different receptor in the cell membrane and leads to a different cell reaction, therefore it is classified as Type II interferon. It could be shown, that IFNγ is produced by $CD8^+$ and $CD4^+$ T-cells [8], natural killer cells [9], endothelial cells and astrocytes [2,3,4]. It is involved in tumor suppression, defense against microbial infections due to macrophage activation, inflammation, inhibition of cellular proliferation, immunmodulation and also viral defense [10,11].

## 1.2 IFNγ receptor

The IFNγ receptor is expressed on virtually any cell type except erythrocytes [12]. It was found that receptor expression in humans has the highest level in nerve, skin and trophoblast cells of the placenta, whereas the expression in immune or hematopoietic cells is much lower [13]. The IFNγ receptor is situated in the cell membrane and is formed by four protein chains. Two of the four chains mainly function as ligand binding chains and are referred as IFN gamma-receptor 1 (IFNγ-R1 or IFNγ-Rα), the other two chains function as signal transducing chains and are referred as IFN gamma-receptor 2 (IFNγ-R2 or IFNγ-Rβ). On the cytosolic side of the receptor, janus kinase 1 and 2 are associated to the receptor (JAK 1 and 2) (Figure 1).

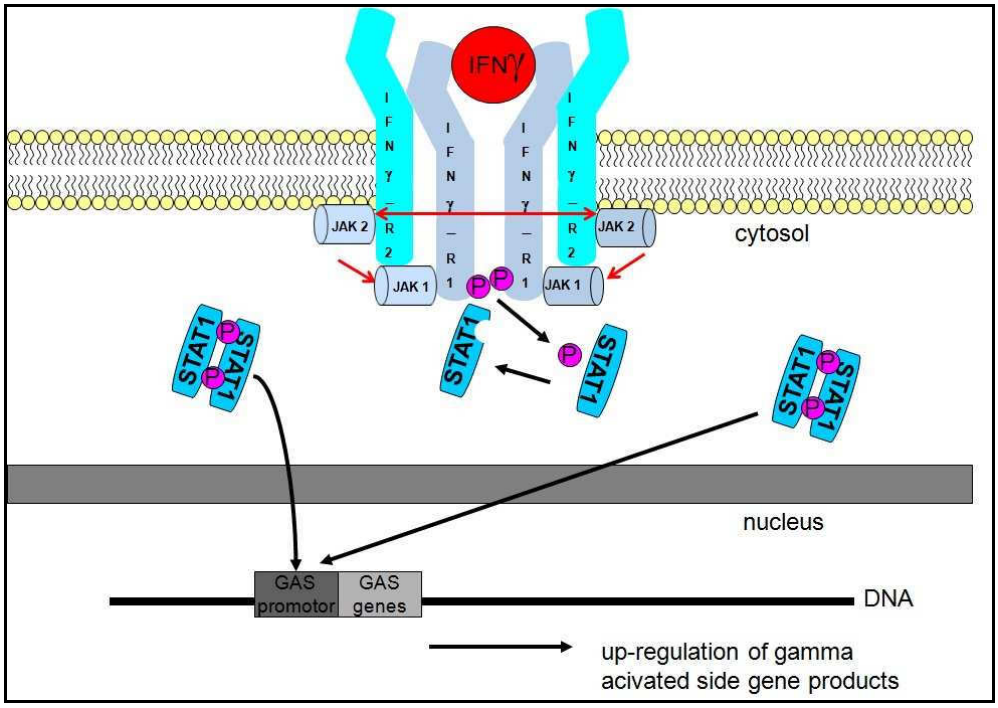


Figure 1: schematic drawing of IFNγ receptor and IFNγ signal transduction

## 1.3 IFNγ signal transduction

The signal transduction of IFNγ takes place over a signal transducing process through members of the janus kinase family (Jak) and the signal transducers and activators of transcription (Stat) family. This pathway is referred as the Jak-Stat pathway, which is a general pathway used by hormones, cytokines and growth factors to regulate the gene-expression of a specific target cell [14]. In the case of IFNγ signaling, the binding of IFNγ to the receptor leads to an autophosphorylation and thus activation of Jak 2 which then leads to a transphosphorylation of Jak 1 [15]. The activated Jak 1 leads to phosphorylation in the IFNγ-R1 chain, so that unphosphorylated Stat 1 molecules can get phosphorylated on the c-terminus of IFNγ-R1 to form phosphorylated dimers, move to the nucleus and then can bind to a promoter sequence [16].

## 1.4 Impact of IFNγ on cells

Nearly all cells in an mammalian organism express receptors for IFNγ, a remarkably set of genes is regulated by IFNγ and IFNγ signaling is not only modulated by this protein itself but by a crosstalk and modulation of other interferons of the IFN type I

family. Thus, a high amount of this protein (due to an infection or an insult) will affect all cells nearby the centre of IFNγ distribution. The effect of IFNγ on cells of the immune system and the following genetic regulations could be enlightened in the past centuries. Thus it could be shown, that IFNγ leads to up-regulation of major histocompatibility complexes (MHC I and II) on the cell surface [17,18,19,20,21,22], antiviral effects [23,24], antiproliferative effects [25,26], apoptosis inducing effects [27,28], reactive oxygen species producing effects [29,30,31], nitrite oxygen species producing effects [32], antimicrobial effects [33,34] and general immunomodulating effects [35,36]. Although the effects of IFNγ on immune cells are well characterised, the impact of IFNγ on cell types of different origin needs further exploration. Especially, since there seems to be a tissue and location specific reaction of cells to IFNγ exposure. In the case of neural stem cells, various studies were aimed at elucidating the role of IFNγ on cell behaviour and revealed plurivalent results. Effects that report IFNγ to conduct neural stem cells into neural differentiation [37,38,39,40,41] stand against reports where IFNγ was reported to elicit negative effects on neurite outgrowth and differentiation behaviour [42,43,44,45,46,47,48].

## 1.5 Stem cells and stem cell potencies

To date, different stem cell types were described and defined by their varying abilities. One characteristic of all stem cell types is the ability to reproduce itself, the other characteristic is, that they can give raise to other cell types and lineages. Adult stem cells build the natural resource of an organism to repair and to replace damaged tissue or cells. In adult organisms, tissue specific stem cells often remain inactive, until they get a signal to proliferate and/or differentiate. In the past, stem cells were divided into three major categories, namely embryonic stem cells, fetal stem cells and adult stem cells. Embryonic stem cells are derived from the inner cell mass of a fertilized egg at the blastocyst stage. The technique to derive these cells was first described for mouse blastocysts in the year 1981 [49] and for human blastocysts in the year 1998 [50]. These cells are referred as pluripotent stem cells (lat. *plurimus* = many; *potens* = potent), since they are able to give raise to ekto-,

meso- and endodermal germlayers of an organism. Multipotent stem cells have the ability to differentiate into all cell types of their specific lineage. Oligopotent stem cells have the ability to differentiate into a few cell types of their specific lineage and unipotent stem cells only can differentiate into one specific cell type. The term totipotent (lat. *totus* = total,entire; *potens* = potent) describes the ability to produce all cells types of a specific organism. Only the fertilized egg is totipotent [51,52,53]. Lately it has been described, that it is also possible to reprogram adult skin fibroblasts into pluripotent cells. These cells are referred as induced pluripotent stem cells (iPSCs). This protocol was first described by Takahashi and colleagues in the year 2006 for mouse cells and in the year 2007 for human cells [54,55].

## 1.6 Stem cells and neurological diseases

Due to their huge potential and restorative properties, stem cells became interesting in a medical context. The possibility to use stem cells as a source for cell replacement in various diseases, as co-stimulators for dormant endogenous stem cells in patients or the usage of transgene cells as carriers for therapeutic agents seems promising. Another important field is the exploration and recapitulation of basal developmental stages and the study of drug administration on neural stem cells. In the field of neurological diseases, cell replacement strategies are another goal. Currently different types of stem cells are used to achieve this. Bone marrow stem cells are transdifferentiated, fetal or embryonic tissue is engineered, adult cells are extracted and stimulated or cells from human embryonic stem cells are differentiated into a specific cell lineage. The currently most promising strategy for cell replacement are iPSCs, because they do not cause donor-host immune reactions. Patient fibroblasts are easy extractable and there are no ethic questions as in the case of human embryonic stem cells. But there are still open questions in the applicability of human iPSC-derived neural cells, since these cells are more or less old and genetic engineering and manipulation was used to produce these cells. Currently several clinical trials are in progress to ascertain the potential of different stem cells in cell transplantation. Fetal human cells were used in Huntington´s disease [56,57,58,59,60,61,62], mesenchymal and porcine cells were used in stroke [63,64], over 300 patients with Parkinson´s disease worldwide were treated with fetal cells

[65,66,67,68] and companies like ReNeuron and Geron are working on further large scale clinical trials and patents. The results of these studies are somewhat promising, but not mature enough for clinical large-scale applications.

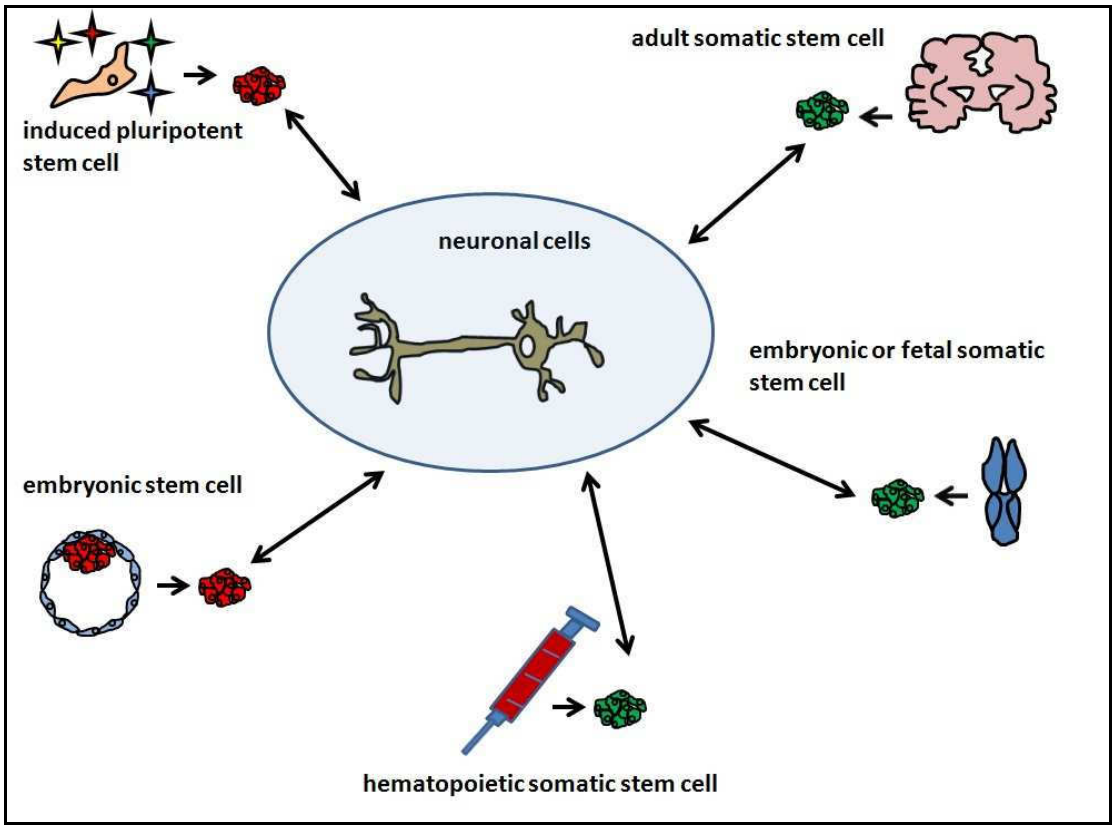


Figure 2: schematic drawing of stem cell types used in neurological research

## 1.7 Stem cell types used in the presented studies

In this study three different cell types were used. The first one consists of murine neural stem/progenitor cells (msNSPCs). These cells are derived from mouse embryos at day 14 after fertilization. The developing brain of the embryo is dissected and the lateral ganglionic eminences are removed and taken into culture. These cells are a mixture of multipotent and oligopotent murine neural stem cells. They are able to proliferate for approx. 15-20 passages until they lose their neurogenic potential. The second cell type consists of murine neural stem cells (msNSCs). They are produced by a serum-free differentiation process from murine embryonic stem cells (mESCs) to murine neural stem cells (msNSCs). This process is driven by different growth factors and cell signaling molecules that were added. The cells that were

produced by this protocol are enriched multipotent neural stem cells. The third cell type that was used in this study consists of human neural stem cells (hESCs). The cell culture principle to produce these cells is analogical to the protocol used for murine embryonic stem cells with some modifications. Human neural stem cells (hNSCs) derived from human embryonic stem cells are multipotent, like murine neural stem cells and can give rise to astrocytes, neurons and oligodendrocytes, after lineage specific differentiation. Allowance of the German government was given to culture and to perform developmental studies with human embryonic stem cells.

# 2. Publications

## 2.1 A new role for interferon gamma in neural stem/precursor cell dysregulation

On the following pages (14-25) the research manuscript “A new role for interferon gamma in neural stem/precursor cell dysregulation” is presented, as published in the journal Molecular Neurodegeneration.

Walter et al. Molecular Neurodegeneration 2011, 6:18
http://www.molecularneurodegeneration.com/content/6/1/18

MOLECULAR NEURODEGENERATION

RESEARCH ARTICLE Open Access

# A new role for interferon gamma in neural stem/precursor cell dysregulation

Janine Walter[1*], Silke D Honsek[2,3], Sebastian Illes[1], Jennifer M Wellen[1], Hans-Peter Hartung[1], Christine R Rose[2], Marcel Dihné[1]

**Abstract**

**Background:** The identification of factors that compromise neurogenesis is aimed at improving stem cell-based approaches in the field of regenerative medicine. Interferon gamma (IFNγ) is a main pro-inflammatory cytokine and up-regulated during several neurological diseases. IFNγ is generally thought to beneficially enhance neurogenesis from fetal or adult neural stem/precursor cells (NSPCs).

**Results:** We now provide direct evidence to the contrary that IFNγ induces a dysfunctional stage in a substantial portion of NSPC-derived progeny *in vitro* characterized by simultaneous expression of glial fibrillary acid protein (GFAP) and neuronal markers, an abnormal gene expression and a functional phenotype neither typical for neurons nor for mature astrocytes. Dysfunctional development of NSPCs under the influence of IFNγ was finally demonstrated by applying the microelectrode array technology. IFNγ exposure of NSPCs during an initial 7-day proliferation period prevented the subsequent adequate differentiation and formation of functional neuronal networks.

**Conclusions:** Our results show that immunocytochemical analyses of NSPC-derived progeny are not necessarily indicating the correct cellular phenotype specifically under inflammatory conditions and that simultaneous expression of neuronal and glial markers rather point to cellular dysregulation. We hypothesize that inhibiting the impact of IFNγ on NSPCs during neurological diseases might contribute to effective neurogenesis and regeneration.

## Background

Neural stem/precursor cells (NSPCs) may be useful as an endogenous or transplantable source of newly generated neural cells, which can replace lost or diseased neurons within the central nervous system (CNS) [1]. A prerequisite for this is an appropriate functional differentiation of immature neural cells into electrophysiologically active neurons. As nearly all CNS diseases involve acute and chronic inflammatory processes [2], it is crucial to understand NSPC development under inflammatory conditions to better realize their full potential. IFNγ is a key inflammatory cytokine, mainly produced by cytotoxic $CD8^+$ T-cells and natural killer cells in the course of neurological diseases like cerebral traumata [3], stroke [4] or multiple sclerosis [5]. Beside the observation that IFNγ-activated microglial cells induce neurogenesis [6], IFNγ has also been reported to exert beneficial, pro-neurogenic effects on NSPC development *in vitro* and *in vivo* in a number of recent publications independently of microglial cells [7-9]. However, a hint that IFNγ might be involved in potentially harmful developmental dysregulation was detected in a number of reports [10-12] and from its tumor-initiating role, since embryonic mice over-expressing IFNγ develop medulloblastomas [13], indicating that IFNγ may also be involved in malignant transformation of neural precursor cells.

In the present study, we demonstrated that IFNγ induces an abnormal immunocytochemical phenotype in NSPCs with simultaneous expression of neuronal and glial markers. Furthermore, IFNγ led to a dysregulated gene expression as well as dysfunctional electrophysiological properties. Additionally, we finally present evidence that IFNγ exposure to NSPCs during an initial 7-day

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proliferation period dramatically impairs the subsequent development of functional neuronal networks as recorded by the microelectrode array technology. Our data clearly indicate that IFNγ compromises neurogenesis. Thus, its role during inflammatory processes should be reassessed and IFNγ suppression during brain pathology possibly supports functional neurogenesis.

## Results

### IFNγ receptors 1 and 2 are expressed in NSPCs and their differentiated progeny

Experiments were performed either with proliferating NSPCs under the influence of growth factors which expressed immature neural markers like Sox2 and nestin (Figure 1A) or with the differentiated progeny of NSPCs that lost their immature markers and instead expressed βIII-tubulin or GFAP (Figure 1B). The signal transduction process of the proinflammatory cytokine IFNγ starts with binding to the IFNγ receptor (IFNGR). This receptor comprises two ligand-binding IFNG-R1 chains which are associated to two signal-transducing IFNG-R2 chains. Both domains of the receptor belong to the class II cytokine receptor family. To study effects of IFNγ on NSPCs and their differentiated progeny, we confirmed the expression of IFNG-R1 and IFNG-R2 in proliferating or differentiated NSPC cultures (Figure 1). We performed immunocytochemical experiments to demonstrate the expression on protein level (Figure 1B). Then we compared the mRNA expression levels of both receptor domains by means of real-time quantitative PCR in various mouse tissues in comparison to proliferative or differentiated NSPCs (Figure 1C). Our results indeed confirmed the presence of both receptor domains in proliferative as well as differentiated NSPCs.

### IFNγ reduces the population extent of NSPCs

To investigate effects of IFNγ on the extent of NSPC populations we performed an MTT-assay. We could

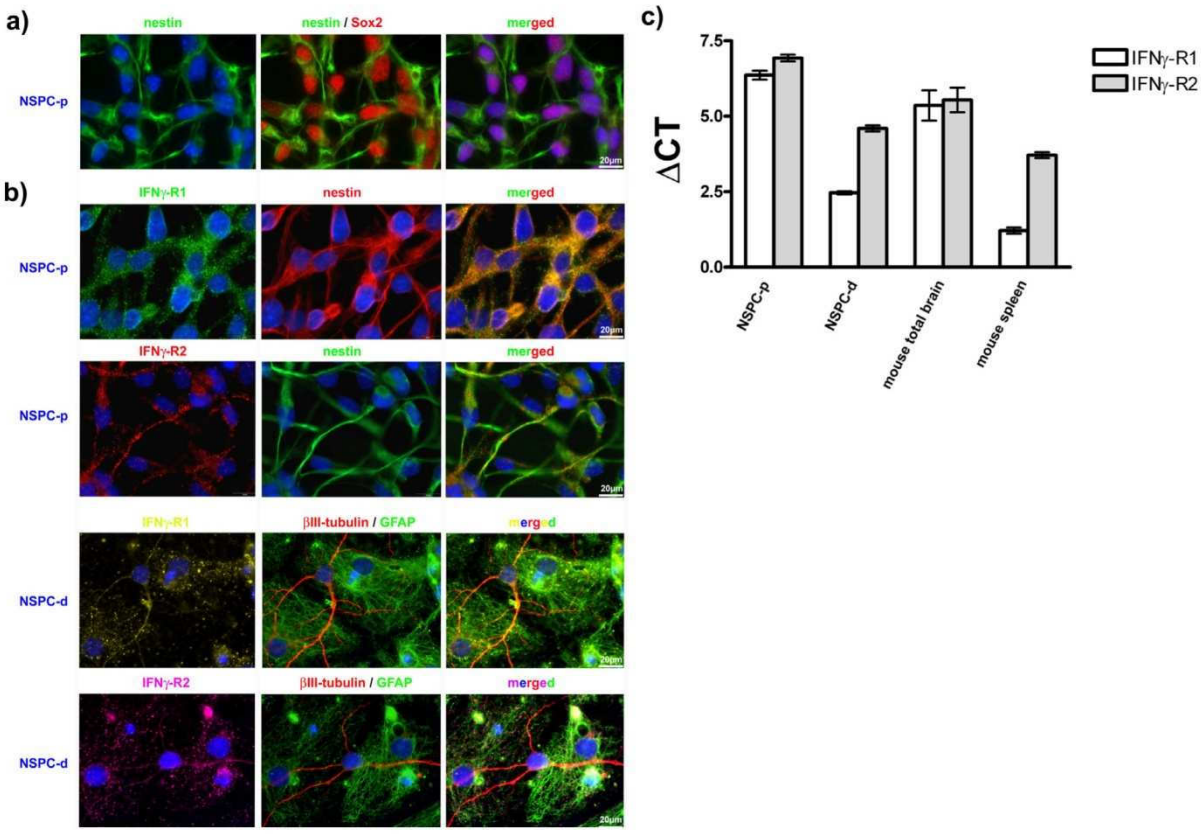


**Figure 1 NSPCs express receptors for IFNγ**. In a: Photomicrographs of proliferating NSPCs (NSPC-p) immunocytochemically labeled against Sox2 and nestin. In b: Photomicrographs of proliferating (NSPC-p) or differentiated (NSPC-d) NSPCs with indicated immunocytochemical markers are given showing that both receptors are expressed on individual cells. In c: real-time quantitative PCR with primers specific for IFNγ-R1 or IFNγ-R2 illustrate the expression of both IFNγ receptors on proliferating (NSPC-p) or differentiated (NSPC-d) NSPCs. Controls are spleen or total brain homogenates. Values are means +/- standard error of mean (SEM). Experiments were performed in triplicate and repeated independently at least three times.

demonstrate that 100 or 1000 Units of IFNγ/ml led to significant reductions in the population extent during 48 hours under proliferative conditions (Figure 2A). To verify whether cytotoxic or apoptotic mechanism were involved, we verified caspase 3/7 activity during IFNγ exposure and found a significant increase when caspase activity was measured by means of the Caspase-Glo 3/7 assay (Figure 2B). Also an increased immunocytochemical labeling against caspase 3/7 protein (Figure 2C) suggested an induction of apoptotic pathways in NSPCs after IFNγ treatment. To detect possible anti-proliferative influences of IFNγ on NSPC populations, we performed BrdU labelings. Here, we were able to detect an additional slight but significant anti-proliferative effect of IFNγ (Figure 2D). Together, these data show that IFNγ exerts apoptotic and anti-proliferative effects on NSPCs that together lead to reduced population extents even under the influence of FGF-2.

#### IFNγ induces an abnormal phenotype in NSPCs

In the present study, we found that IFNγ treatment of proliferative murine E14 neurosphere-derived NSPCs caused up-regulation not only of transcripts for βIII-tubulin or microtubule-associated protein 2a-c (MAP2a-c), established markers for post-mitotic neurons, but also for the astrocyte marker glial fibrillary acidic protein (GFAP), challenging the prevalent view of a predominatly pro-neurogenic effect of IFNγ. At the same time, IFNγ executed a down-regulation of CD133, a marker for immature NSPCs, which indicated a robust activation of differentiation programs despite the presence of growth factors (Figure 3A). Immunocytochemical experiments confirmed these results: on the one hand, we could detect an anti-proliferative effect of IFNγ as numbers of $BrdU^+$ cells decreased (Figure 2D). On the other hand, we detected a robust increase in the number of cells expressing neuronal and glial specific proteins after IFNγ treatment (Figure 3B). Surprisingly, in addition to $GFAP^-$/βIII-$tubulin^+$ or $GFAP^+$/βIII-$tubulin^-$ cells, a considerable number of NSPCs (39.3 ± 14.5% of all cells) co-expressed GFAP and βIII-tubulin after a 3-day treatment with 1000 U/ml IFNγ (Figure 3B). $GFAP^+$/βIII-$tubulin^+$ cells in comparable numbers were also detectable after IFNγ treatment with only 100 U/ml (Figure 3B). Using a concentration of 100 U/ml IFNγ led to a slightly weaker induction of GFAP and βIII-tubulin immunoreactivity in individual cells while numbers of cells showing at all this phenomenon were similar with 100 or 1000 U/ml. This phenomenon was absolutely rare (< 0.01%) in the absence of IFNγ (Figure 3B). In addition, IFNγ treatment induced simultaneous expression of GFAP and the post-mitotic neuronal markers MAP2a-c in a large percentage of cells ($GFAP^+$/$MAP2a\text{-}c^+$: 73.6 ± 5.7%), illustrating that the co-expression of glial and neuronal markers is not restricted to βIII-tubulin. We next investigated the influence of IFNγ treatment during a 7-day differentiation period after growth factor withdrawal from NSPC cultures to elucidate effects on cell maturation. Again, we detected cells co-expressing GFAP and βIII-tubulin (Figure 4A). Moreover, numbers of $GFAP^-$/βIII-$tubulin^+$ or $GFAP^+$/βIII-$tubulin^-$ cells in IFNγ treated cultures differentiated for 7 days were significantly lower than without IFNγ Figure 4A), which is in direct contrast to the expected pro-neurogenic role of IFNγ. As terminal neuronal differentiation could take longer than 7 days, we also cultured NSPC populations without growth factors for 14 or 21 days under the influence of IFNγ. Also here we found $GFAP^+$/βIII-$tubulin^+$ cells indicating that this phenotype is stable during 14 or 21 days under differentiation conditions (data not shown). To verify if this phenomenon depends on more restricted precursors being present in neurosphere-derived populations, we generated homogenous cultures of multipotent neural stem cells (NS cells). These populations were generated from murine embryonic stem cells. Also in NS cell cultures, $GFAP^+$/βIII-$tubulin^+$ cells were induced even though the part of NS cell-derived $GFAP^+$/βIII-$tubulin^+$ cells was smaller in comparison to neurosphere-derived $GFAP^+$/βIII-$tubulin^+$ cells. This shows that IFNγ can generate this abnormal phenotype from immature neural stem cells independently of the presence of neuronal or glial precursors (Figure 4B). Thus, our experiments suggest that IFNγ can drive the differentiation of NS cells or NSPC populations towards an immunocytochemically abnormal marker profile, indicating a genetic and/or functional dysregulation.

#### IFNγ induces an abnormal down-stream signaling in NSPCs

To study changes in IFNγ receptor expression, we performed quantitative real-time PCR after a 3-day IFNγ treatment of proliferative NSPCs. We detected increases in the transcript numbers of IFNγ-receptor1 and IFNγ-receptor2 (Figure 5A), as well as the IFNγ-related downstream factor signal transducers and activators of transcription 1 (Stat1). However, inducible nitric oxide synthase (iNOS), a gene product, which is usually up-regulated as a result of IFNγ signaling, was down-regulated, revealing another surprising effect of IFNγ on NSPCs (Figure 5A). We investigated IFNγ-induced changes of pro-neural basic helix-loop-helix (bHLH) genes and the neurogenic transcription factor Pax6, which are important for neuronal determination. Supporting the notion that IFNγ does not promote neuronal determination, we found that Math1, Mash1, Neurogenin1 and Pax6 were down-regulated in NSPCs after IFNγ treatment (Figure 5B). Further, while IFNγ

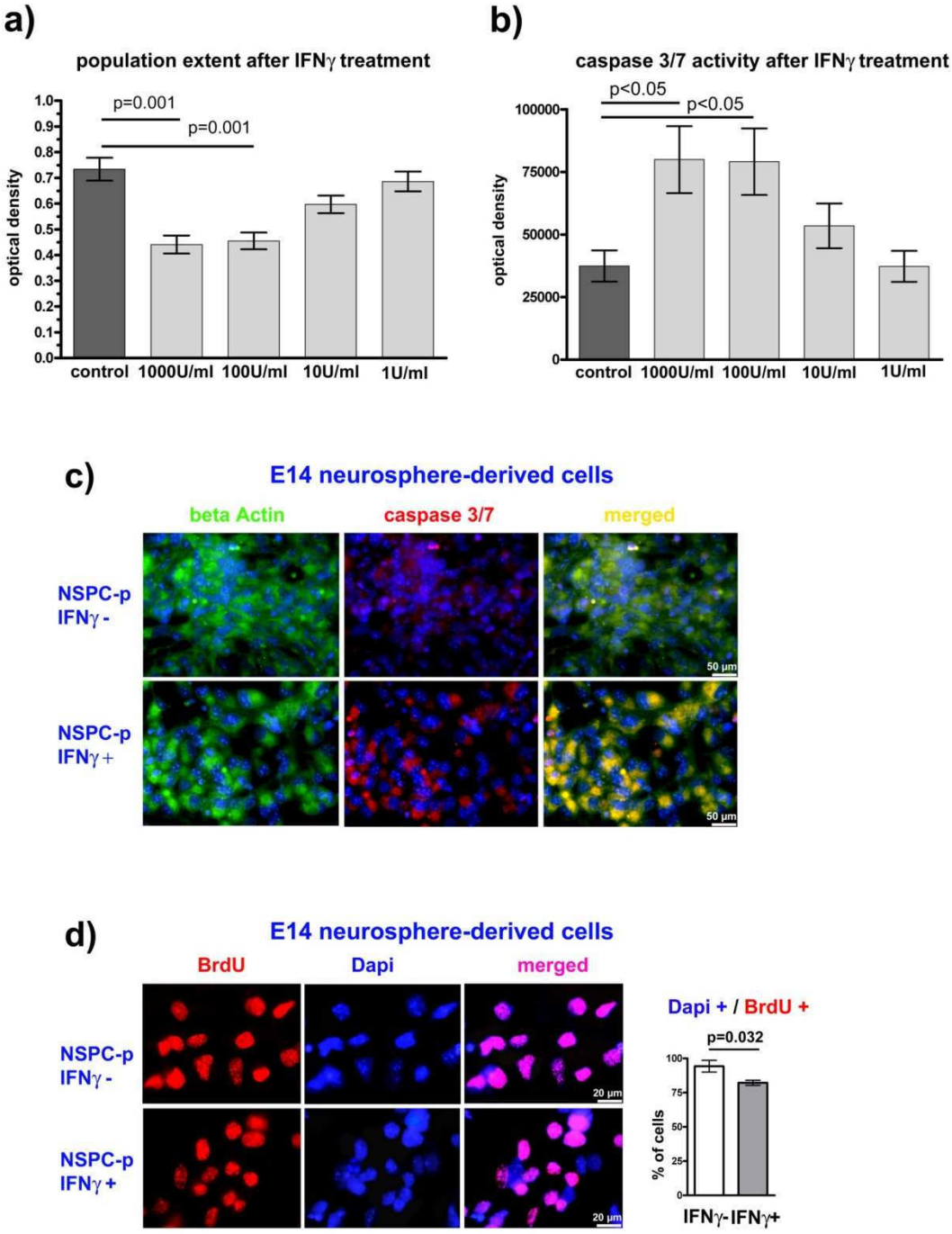


**Figure 2 IFNγ reduces the population extent of NSPCs**. In a: Optical densities [9] correlate to the extent of NSPC populations after a 48-hour IFNγ treatment with indicated concentrations. Values are means +/- standard error of mean (SEM). Experiments were performed in triplicate and repeated independently at least three times. In b: Optical densities correlate to caspase 3/7 activity after a 48-hour IFNγ treatment with indicated concentrations. Values are means +/- standard error of mean (SEM). Experiments were performed in triplicate and repeated independently at least three times. In c: Photomicrographs of caspase 3/7 immunocytochemistries are given for proliferating (NSPC-p) NSPCs with or without IFNγ treatment. Beta Actin labeling visualizes cytoplasmatic structures to better correlate the caspase 3/7 signal to single cells. In d: Photomicrographs of BrdU labelings are given to visualize the amount of proliferating cells with or without IFNγ treatment. Additionally, quantification of $BrdU^+$ cells is given. Values are means +/- standard error of mean (SEM). Experiments were performed in triplicate and repeated independently at least three times. $DAPI^+$ nuclei are given in blue.

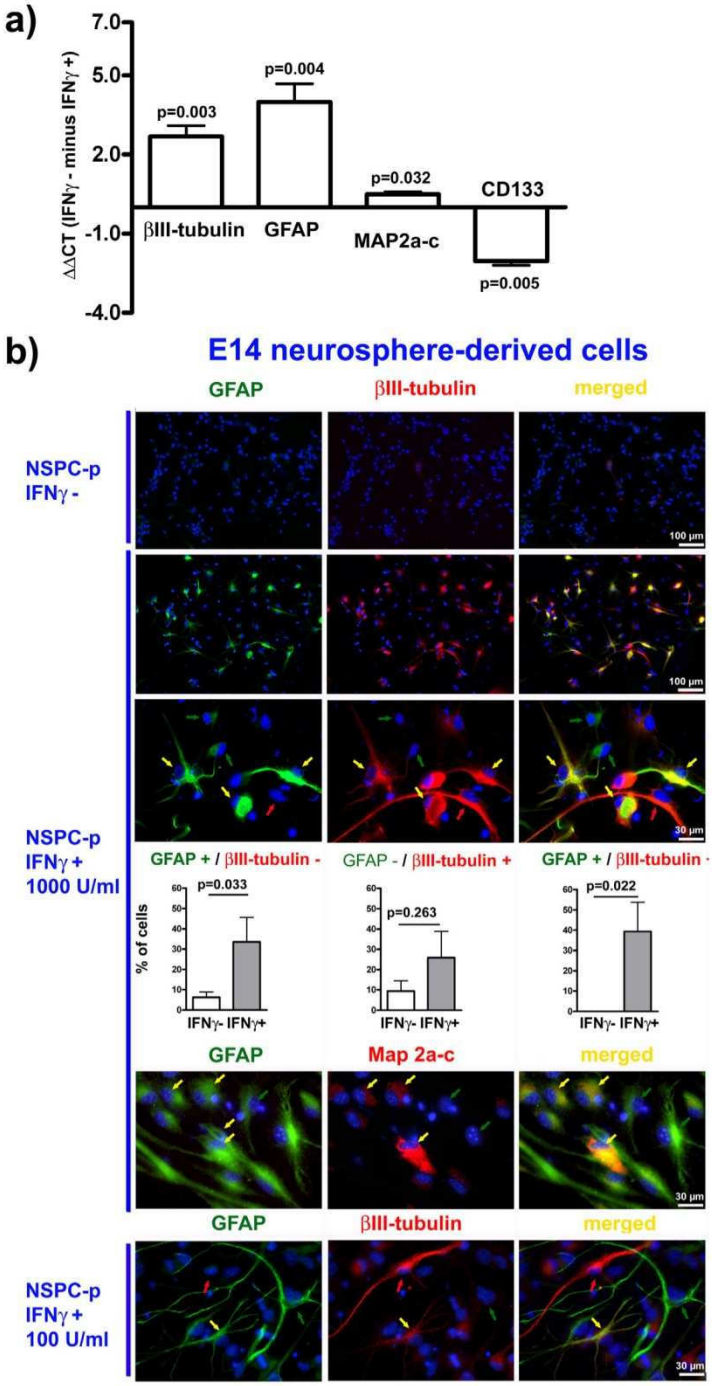


**Figure 3 Cell type-specific marker expression after IFNγ treatment under proliferative conditions**. In a: Results from real-time quantitative PCRs expressed as ΔΔCT (IFNγ- minus IFNγ+) of PBS-treated control and IFNγ-treated groups for the indicated markers of proliferating NSPCs. Higher values indicate a higher gene expression. Values are means +/- standard error of mean (SEM). Experiments were performed in triplicate and repeated independently at least three times. In b: Photomicrographs of proliferating NSPCs (NSPC-p) with indicated immunocytochemical markers (GFAP, βIII-tubulin, Map2a-c). Yellow arrows mark GFAP$^+$/βIII-tubulin$^+$ cells, the red arrow marks a GFAP$^-$/βIII-tubulin$^+$ neuron and green arrows mark GFAP$^+$/βIII-tubulin$^-$ astrocytes. DAPI$^+$ nuclei are given in blue. Diagrams show the percentages of immuno-positive cells from all DAPI$^+$-cells with (IFNγ+) or without (IFNγ-) IFNγ treatment as indicated. Values are means +/- standard error of mean (SEM). Experiments were performed in triplicate and repeated independently at least three times.

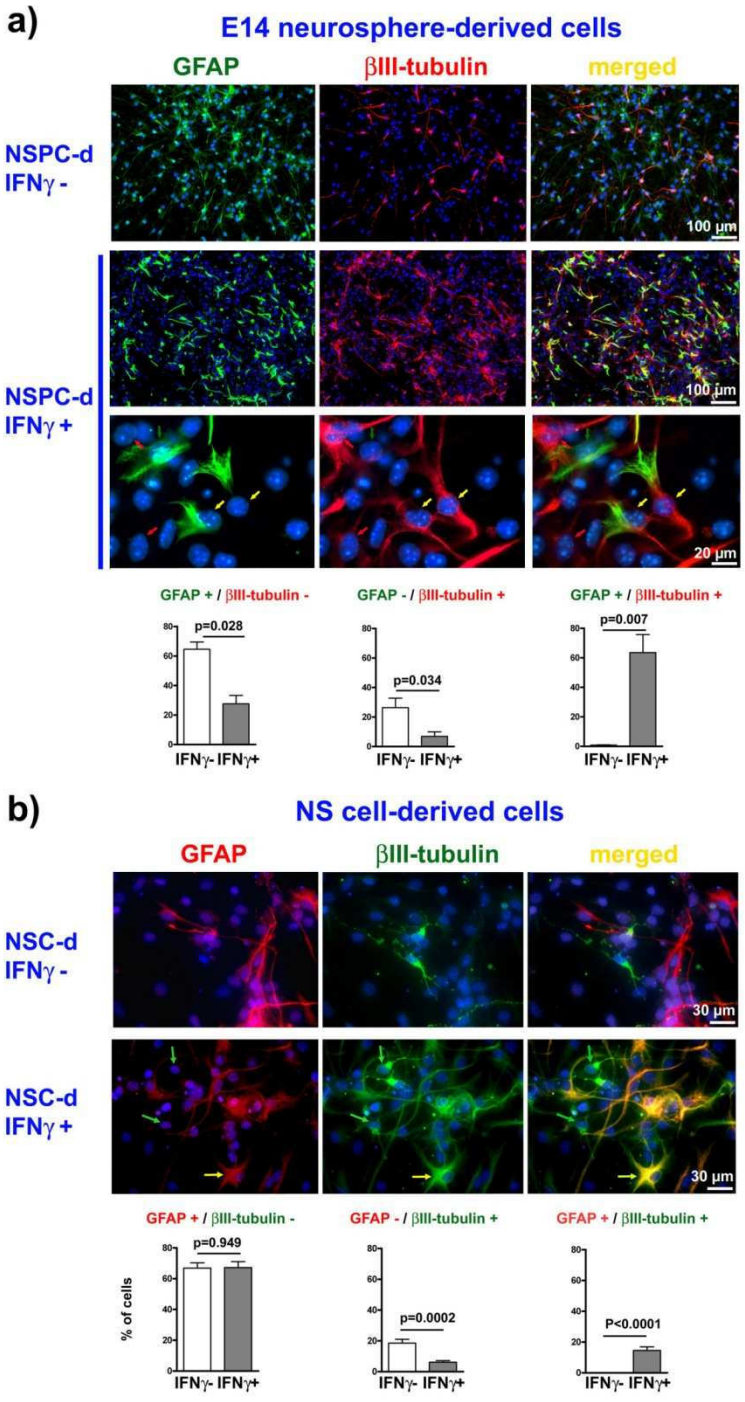


**Figure 4 Cell type-specific marker expression after IFNγ treatment during differentiation**. In a: Photomicrographs of NSPC populations after a differentiation period of 7 days (NSPC-d) are given with indicated immunocytochemical markers and the corresponding quantification of immuno-positive cells from all DAPI$^+$ cells with (IFNγ+) or without (IFNγ-) IFNγ treatment as indicated. Values are means +/- SEM. Experiments were performed in triplicate and repeated independently at least three times. In b: Photomicrographs of mouse embryonic stem cell-derived neural stem cells (NS cells) differentiated for 7 days (NSC-d) with indicated immunocytochemical markers with (IFNγ+) or without (IFNγ-) IFNγ treatment are given. Yellow arrows mark GFAP$^+$/βIII-tubulin$^+$ cells. Red arrows mark GFAP$^+$/βIII-tubulin$^-$ neurons and green arrows mark GFAP$^-$/βIII-tubulin$^+$ astrocytes. Note the untypical morphology of GFAP$^+$/βIII-tubulin$^+$ cells under IFNγ treatment in comparison to control. Values are means +/- standard error of mean (SEM). Experiments were performed in triplicate and repeated independently at least three times.

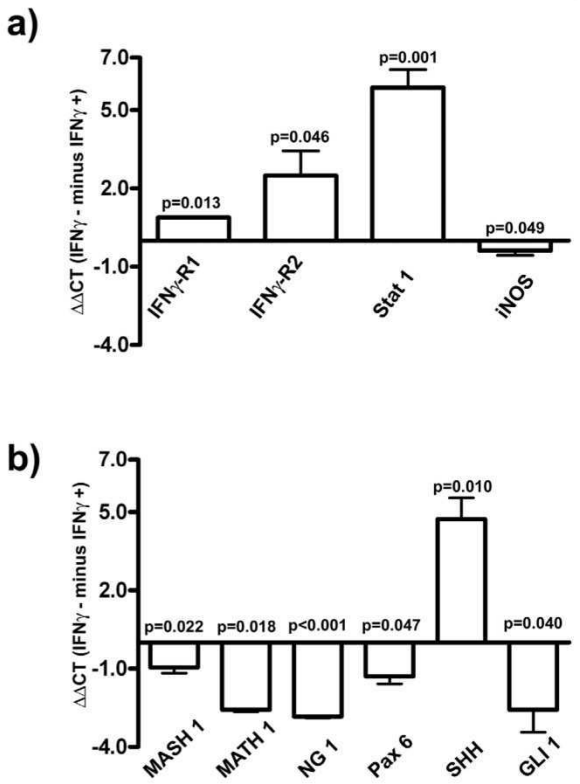


**Figure 5 Down-stream signaling analysis after IFNγ treatment under proliferative conditions**. In a and b: Results from real-time quantitative PCRs expressed as ΔΔCT (IFNγ- minus IFNγ+) of PBS-treated control and IFNγ-treated groups for the indicated markers of proliferative NSPCs. Higher values indicate a higher gene expression. Values are means +/- standard error of mean (SEM). Experiments were performed in triplicate and repeated independently at least three times.

treatment significantly up-regulated SHH in NSPCs, Gli1 was down-regulated, an effect that was unanticipated (Figure 5B).

### GFAP$^+$/βIII-tubulin$^+$ cells exhibit non-neuronal and non-astrocytic functional properties

To elucidate if the unusual effects of IFNγ treatment on the differentiation of NSPCs are accompanied by an atypical functional phenotype, we next analyzed basic electrophysiological properties of GFAP$^+$/βIII-tubulin$^+$ cells. For this purpose we combined whole-cell patch-clamp with subsequent immunocytochemistry to unambiguously identify recorded cells. GFAP$^+$/βIII-tubulin$^+$ cells in IFNγ-treated proliferative (n = 5) or differentiated (n = 9) cultures almost exclusively exhibited an outward rectifying current-voltage (IV) relationship (13/14 cells, Figure 6a-c). Small inward currents were observed in 4/14 cells (Figure 6d). When challenging GFAP$^+$/βIII-tubulin$^+$ cells within differentiated cultures with current injections in current-clamp mode, none of these cells exhibited action potentials when depolarized to up to a membrane potential of approximately +30 mV (n = 7, Figure 6e, f). GFAP$^+$/βIII-tubulin$^-$ cells in differentiated cultures either exhibited an outward rectifying IV relationship, similar to GFAP$^+$/βIII-tubulin$^+$ cells (3/6; Figure 6g, h, i), or a linear IV-relationship that completely lacked voltage-dependent conductances (n = 3/6; Figure 6g, h, i). Such I/V relationships are typical for mature classical astrocytes [14] and were never observed in GFAP$^+$/βIII-tubulin$^+$ cells. Our electrophysiological results, thus, demonstrate that GFAP$^+$/βIII-tubulin$^+$ cells are functionally distinct from mature astrocytes as well as neurons.

### IFNγ treatment impaired the formation of in vitro-functional neural networks

To ultimately verify the effect of IFNγ on NSPC populations, we performed experiments using the microelectrode array (MEA) technology, that is able to detect functional neuronal network activity of entire neural populations. Immature ES cell-derived nSFEB aggregates consisting of a mixture of immature neural precursor cells were exposed to IFNγ during their initial 7-day proliferation period under the influence of FGF-2. Hereafter, IFNγ was removed and differentiation was initiated by FGF-2 withdrawal. By this paradigm, IFNγ treatment selectively hit developmental processes of ES cell-derived NSPCs, while subsequent synapse formation and other maturational processes were excluded from direct IFNγ influences. After the initial 7-day period, maturation of cultures was observed for additional 44 days. Normally, during the 3$^{rd}$ and 4$^{th}$ week, cultures start to develop functional neuronal networks that show increasing burst activity that finally ends in oscillating and synchronous neuronal network activity (Figure 7, IFNγ-). This synchrony of action potential bursts in spatially distributed neurons is expressed by the kappa value, with increasing values showing higher network synchrony. By this experiment we could show that a 7-day exposure to IFNγ during a developmentally sensitive period of immature NSPCs under the influence of FGF-2 sustainably impairs the subsequent generation of functional neuronal networks as burstrate and kappa levels were significantly smaller in comparison to untreated populations (Figure 7). As neuronal network formation can also be impaired by low cell densities, we verified this factor under IFNγ-treated conditions. However, even though IFNγ led to a reduced population extent, the 7-day proliferation period was by far sufficient to allow for the growth of morphologically dense and confluent neural cell populations.

## Discussion

Our results shed new light on the effects of IFNγ on NSPCs. Until now, IFNγ-related up-regulation of

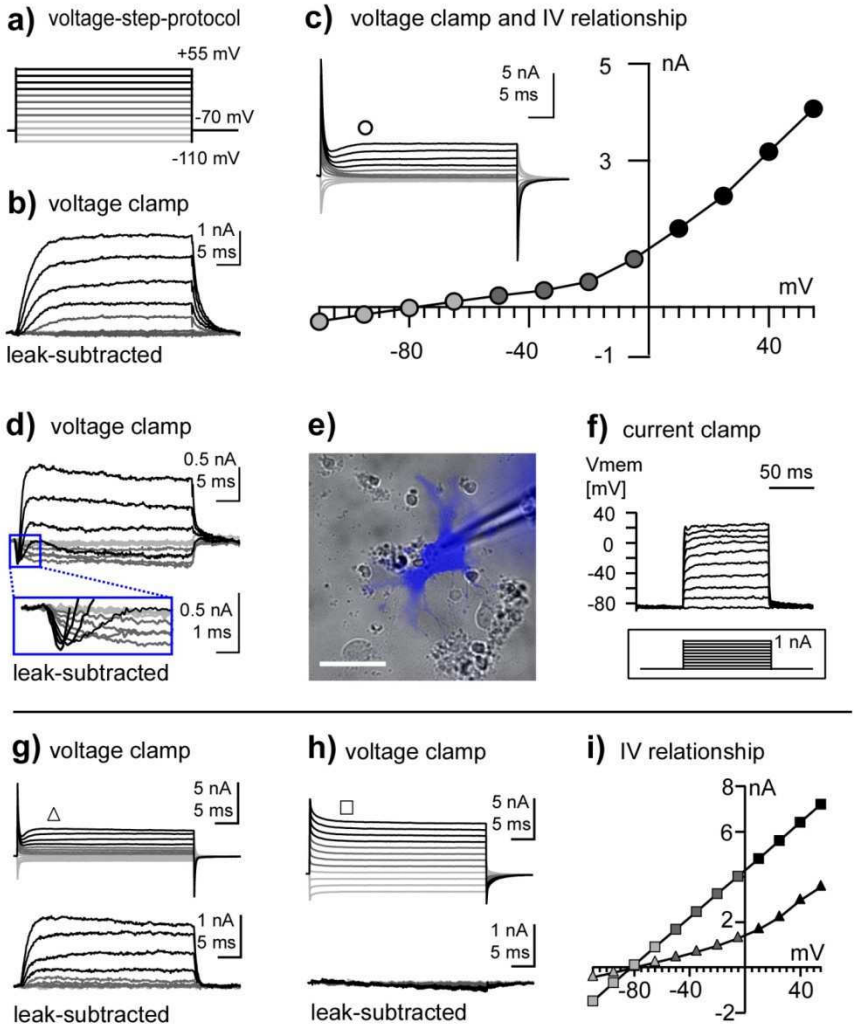


**Figure 6 Patch-clamp results of IFNγ treated NSPCs**. In a: Voltage-step protocol used to probe cells for voltage-activated conductances (see current traces in panels b-d, g, and h). In b, c: GFAP$^+$/βIII-tubulin$^+$ cells typically exhibited an outward rectifying current voltage (IV) relationship (13/14 cells), IV-relationship was determined from non-leak-subtracted traces 10 ms after the voltage step (circle). In d: In 4/14 GFAP$^+$/βIII-tubulin$^+$ cells, voltage-gated inward currents were observed in addition to outward currents. In e: Image of cell filled with Alexa Fluor350 and later identified as GFAP$^+$/βIII-tubulin$^+$ (scale bar 30 μm, recordings illustrated in figure d) and f)). In f: None of the 14 GFAP$^+$/βIII-tubulin$^+$ cells exhibited action-potential-like events, upon depolarization to at least -30 mV evoked by current injections (inset). In g, h: Membrane currents typically observed in GFAP$^+$/βIII-tubulin$^-$ cells (each recorded in 3/6 cells). In i: IV-relationships taken from non-leak-subtracted traces illustrated in g) (triangle) and h) (square).

βIII-tubulin was interpreted as a beneficial enhancement of neurogenesis [7-9]. The present study disproves this view and shows that IFNγ instead promotes an abnormal NSPC-derived cellular phenotype that does not relate to classical neurons or astrocytes and that appears to be dysregulated in terms of functional and molecular properties. IFNγ treatment leads to the expression of both, class III βtubulin and GFAP in ~40% of NSPC which is abnormal and, even after differentiation, not linked to mature neuronal or astrocytic electrophysiological function. Class III βtubulin isotype is usually considered specific for post-mitotic neurons, and such aberrant expression has so far only been noted in gliomas [15,16] or dysregulated tumorigenic neural stem cells [17,18]. Walton and colleagues even report some unusual co-expression of βIII-tubulin and GFAP in tumorigenic neural stem cells, a phenomenon similar to that detected here after IFNγ treatment of regular NSPCs. The aspect of IFNγ-mediated NSPC dysregulation is further substantiated in the present report by an up-regulation of SHH which is paralleled by down-regulation of Gli1 which has been reported to be consistently up-regulated in the

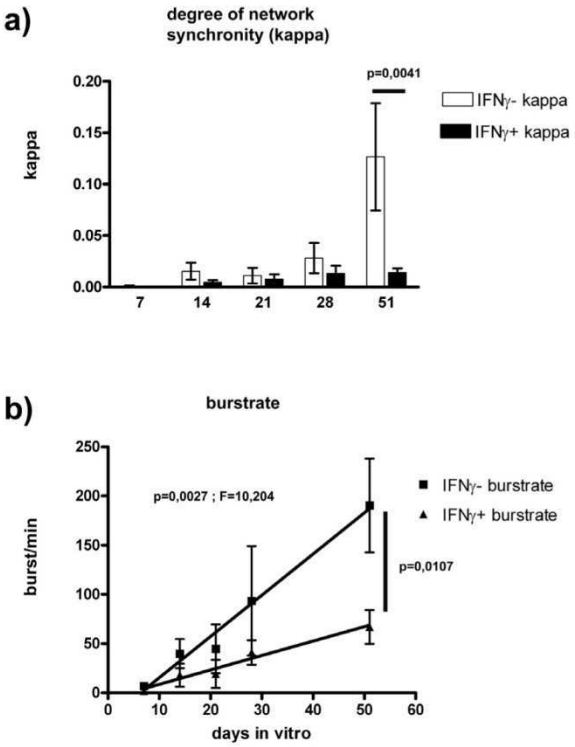


**Figure 7 MEA-recordings of mouse ES cell-derived neural populations**. In a + b: Different electrophysiological parameters of MEA-recordings with or without IFNγ treatment in differentiated mouse ES cell-derived neural precursor cells at indicated time points are illustrated. In a: The synchrony of the neural network which is expressed in kappa values. In b: Bursts per minute are given. In a, the student´s t-test is applied. In b, linear regression is given with p- and F-values and, additionally, the t-test is performed for the 51 days in vitro time point. Values are means +/- standard error of mean (SEM). Experiments were performed in triplicate and repeated independently at least three times.

course of SHH signaling [19]. As expression patterns of neurogenic niche morphogenes like SHH or Gli1 are generally tightly regulated during CNS development, its disturbance points to misguided development or again tumorigenesis [13,19]. Thus, for the first time, we directly illustrated a possible link between IFNγ, NSPCs and cellular abnormalities similar to that observed in tumor cells strongly supporting the view that inflammation might be involved in tumor generation via neural stem cells. Additionally, the IFNγ-related down-regulation of iNOS in NSPC cultures is untypical as it is known that IFNγ normally induces iNOS [20]. Our electrophysiological findings illustrate the importance of an additional functional control of morphological/immunocytochemical observations as the up-regulation of βIII-tubulin in differentiated NSPC-derived cells, which was interpreted as enhanced neurogenesis in different studies [7,9], was not paralleled by neuronal electrophysiological behavior. Further, the increase in $GFAP^-/\beta III\text{-}tubulin^+$ neurons after IFNγ treatment of proliferating cultures was not significant in the present study and after differentiation under the impact of IFNγ we even found significantly less $GFAP^-/\beta III\text{-}tubulin^+$ neurons. Interestingly, a similar observation was described previously [21]. We found that those βIII-tubulin expressing cells that significantly increased in numbers after IFNγ treatment of proliferating or differentiating cultures were also GFAP positive and exhibited electrophysiological properties that were neither typical for mature astrocytes nor for neurons. We demonstrated this by careful correlating electrophysiological data of patched cells with their immunocytochemical phenotype. These molecular and functional IFNγ effects on NSPCs indicate a profoundly compromised cell function or, alternatively, a new IFNγ-induced NSPC-derived neural cell of unknown function. Interestingly, ectopic expression of IFNγ during early stages of CNS development induces medulloblastomas via SHH overexpression [13] pointing towards a general dysregulating effect of IFNγ on NSPCs during development or disease.

To investigate functional neural development under controlled conditions, with and without IFNγ-treatment, we electrophysiologically measured the development of functional neuronal networks starting from ES cell-derived immature neural precursor cell cultures. Usually, network activity progressively develops over time as a result of a complex interaction of a multitude of factors that converge to an integrated functional entity [22]. It depends on efficient synapse formation and function of an entire neuronal population. If using immature neural precursor populations as developmental starting point, basic aspects of functional neural development can be measured. In contrast, mature ES cell-derived functional neuronal networks can be used to detect acute functional consequences due to changes in extracellular composition. These investigations then affect already active neuronal networks. For instance, they showed to reversibly alter their network function under the influence of different cerebrospinal fluid specimens [23]. We chose a paradigm in which the influence of IFNγ selectively affected the initial proliferation period of cultures that were subsequently held under normal differentiating conditions. IFNγ-treated cultures showed a significantly impaired development of neuronal network function, impressively pointing to an IFNγ-related, profoundly altered functional development of neural precursor populations.

## Conclusion

Thus, we speculate that abnormally high IFNγ production during development and CNS diseases might impair

functional neuronal development in fetal neurogenesis or adult regeneration and propose to inhibit IFNγ effects on NSPCs as a means to effectively support their developmental and regenerative potential.

## Materials and methods

### Neurosphere cultures

Neurospheres were generated from fourteen-day-old wild-type C57BL/6J mouse embryos. Ganglionic eminences were removed, mechanically dissociated and seeded in DMEM/F12 culture medium (1:1; Invitrogen, Karlsruhe, Germany) containing 0.6% Glucose (Sigma-Aldrich, Hamburg, Germany), glutamine (2 mM; Invitrogen), sodium bicarbonate (3 mM; Invitrogen), Hepes buffer (5 mM; Invitrogen) and B27 (20 μl per ml; Invitrogen). For generation and expansion of neurosphere cells, epidermal growth factor (EGF) (Tebu-bio, Le Perray en Yvelines Cedex, France) and basic fibroblast growth factor-2 (FGF-2) (Tebu-bio) were added to a final concentration of 20 ng per ml each.

### Generation of embryonic stem cell-derived neural stem cells

Undifferentiated ES cells (SV-129, ATCC, Millipore, Billerica, USA) were grown under feeder-deprived conditions in the presence of 1000 U/ml leukemia inhibitory factor (LIF, Millipore) and 20% fetal bovine serum (FBS, HyClone, Thermo Fisher Scientific, Schwerte, Germany) in ES cell medium described elsewhere [24]. Neural differentiation of immature ES cells into neural stem cell (NS cells) was performed according to modified protocols [22,25].

### IFNγ treatment and immunocytochemistry

For immunocytochemistry, neurosphere cells or ES cell-derived NS cells were dissociated to a single cell suspension and plated on poly-L-ornithine (PLO; 0.001%; Sigma-Aldrich) and fibronectin (5 μg/ml; Tebu-bio) coated cover slips (VWR International, Darmstadt, Germany) at a density of $50 \times 10^3$ cells per ml. After 3 days under the influence of EGF and FGF-2 (20 ng/ml both Tebu-bio), cells were assigned to the different experimental groups. To verify the marker expression of undifferentiated (proliferating) neural populations under control or IFNγ treated conditions, cultures were kept under the influence of EGF/FGF-2 without or with IFNγ (1000 U/ml; Millipore) until fixation after further 3 days (NSPC-p -IFNγ/+IFNγ). To verify cell-type specific marker expression in differentiated cultures, growth factors were withdrawn and then, cells were treated for 7 days without or with IFNγ until fixation (NSPC-d -IFNγ/+IFNγ). For control experiments, only phosphate-buffered saline solution (PBS; 1X; Invitrogen) was added to the medium. Primary antibodies used at 4°C overnight were monoclonal mouse antibodies to 5-bromo-2-deoxyuridine (BrdU; 1:1000, Sigma-Aldrich), βIII-tubulin (Tuj1; 1:500; R&D Systems, Minneapolis, USA or 1:800, Abcam, Cambridge, UK), Map2a-c (1:2000; Sigma-Aldrich), Sox2 (1:50; R&D Systems), IFNγ-R1 (1:500; Santa Cruz Biotechnology) and beta Actin (1:100; Millipore) and polyclonal rabbit antibodies to glial fibrillaric acid protein (GFAP) (1:500; Dako, Hamburg, Germany or 1:1000; Abcam), caspase (1:100; Cell Signaling), IFNγ-R2 (1:500; Santa Cruz Biotechnology) and nestin (1:200; Covance). BrdU labeling is described elsewhere (Wellen et al., 2009). For detection of primary antibodies, fluoresceine-isothiocyanate (FITC; 1:500; Millipore) and indocarbocyanine (Cy3; 1:800; or Cy5; 1:200; Millipore) coupled secondary antibodies were used. For negative controls, primary antibodies were omitted in each experiment. To measure the total population of cells, Dapi positive cell nuclei were counted. On every cover slip, at least 100 cells were counted.

### MTT-Assay

To analyze the population extent of NSPCs, the optical density [9], indicative of conversion of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) into formazan crystals which takes place in live cells only, was determined after IFNγ treatment at decreasing concentrations as indicated. An OD value of 0.5 represents approximately 50,000, and an OD value of 1.0 represents approximately 100,000 live NSPCs. The population extent was measured after 48 hours of IFNγ treatment as indicated.

### Caspase-activity Assay

For detection of caspase 3/7 activity after IFNγ treatment, we used the Caspase-Glo 3/7 assay (Promega, Madison, USA). Proliferating cultures were treated with decreasing concentrations of IFNγ as indicated. Adding the assay components to cultivated cells leads to cell lysis and release of caspase 3/7. Caspase 3/7 is capable of cleaving a tetrapeptide sequence substrate; this is dismantled by luciferase which is a component of the assay. The resulting light emission is then a measure of caspase activity.

### Quantitative real-time PCR

RNeasy Kit (Qiagen) was used for RNA isolation of cultured NSPCs. Then a reverse transcription into cDNA (ABI, Darmstadt, Germany) was performed. Quantitative real-time PCR was carried out by the usage of the 7500 fast or 7500 quantitative real-time PCR cycler (ABI, Darmstadt, Germany). Either SYBR green master mix

(Qiagen) or equivalent chemistry from another supplier (Quantace, London, UK) was used. The specific primer for genes of interest or the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) was either purchased (QuantiTect primer assays, Qiagen) or self designed (BioTEZ, Berlin, Germany). The genes of interest (target gene) in IFNγ-treated groups or control groups (PBS-treated) were analyzed in at least 3 independent cultures in triplicate each. Every experiment in IFNγ-treated or control (PBS-treated) groups provided delta CT values (ΔCT: gene of interest minus housekeeping gene). The presented graphs are ΔΔCT values:

$$\Delta\Delta CT = (Ct_{\text{target gene}} \quad Ct_{\text{housekeeping gene}})_{\text{PBS-treated}} - (Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}})_{\text{IFN}\gamma\text{-treated}}$$

### Patch-clamp recordings

Somatic whole-cell patch-clamp recordings were carried out using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) coupled to a personal computer via a digidata 1322A interface (Molecular Devices). Data were acquired at 10 kHz using PClamp 8.2 software (Molecular Devices). Patch pipettes were pulled from borosilicate glass (Hilgenberg, Waldkappel, Germany) and had a resistance of 3-6 MΩ when filled with intracellular solution containing (in mM): 120 K-$MeSO_3$, 32 KCl, 10 HEPES (N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid), 4 NaCl, 4 Mg-ATP and 0.4 Na-GTP, 1 Alexa Fluor 350 (Molecular Probes/Invitrogen), pH 7.30 (calculated liquid junction potential: 12.5 mV). Cells were held at membrane potentials of -70 mV. To separate passive conductances from voltage-gated currents, online leak subtraction (P/4) was performed. Extracellular solution during patch-clamp experiments contained in mM: 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 $NaH_2PO_4$, 26 $NaHCO_3$, and 20 glucose, bubbled with 95% $O_2$ and 5% $CO_2$ to result in a pH of 7.4. Patch-clamp data were processed and analyzed using IGOR Pro-Software (WaveMetrics, Inc., Lake Oswego, OR). After the recordings, patch-pipettes were carefully withdrawn and coverslips were transferred into 4% paraformaldehyde for 20 minutes at room temperature. Thereafter coverslips were kept in PBS (Invitrogen) at 4 °C until they were processed for GFAP and βtubulin immunocytochemistry. By means of fluorescence at 350 nm electrophysiologically recorded cells were identified and assigned either to $GFAP^+$/βIII-$tubulin^+$ or $GFAP^+$/βIII-$tubulin^-$ cells.

### Microelectrode array recordings

For microelectrode array (MEA) recordings, 5 to 10 neural precursor cell-enriched, serum-free, floating embryoid body-like aggregates (nSFEBs) [22] were seeded on poly-D-lysine (PDL, 15 μg/ml, Sigma-Aldrich, Germany) and laminin (15 μg/ml, Sigma-Aldrich, Germany) coated MEAs with a square grid of 60 planar Ti/TiN electrodes (30-μm diameter, 200-μm spacing) and an input impedance of <50 kÙ according to the specifications of the manufacturer (Multi Channel Systems, Reutlingen, Germany). Signals from all 60 electrodes were simultaneously sampled at 25 kHz, visualized and stored using the standard software MC Rack provided by Multi Channel Systems. Spike and burst detection was performed off-line by custom-built software (Result, Düsseldorf, Germany). nSFEBs were kept for 7 days after plating under the influence of FGF-2 (20 ng/ml, PeproTech) only (IFN-γ - group) or together with IFN-γ (1000 U/ml; IFN-γ + group). After 7 days, FGF-2 and IFN-γ were removed from the medium to induce terminal differentiation. For long-term culture, ES cell-derived neuronal networks were kept in DMEM/F12 (Gibco) supplemented with N2, B27 and Glutamax (all Invitrogen). MEA recordings were performed at the indicated time points.

### Statistical analyses

Experiments were repeated with independent cultures at least three times in triplicate each. The resulting data sets were statistically analyzed und illustrated using the GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA, 2003) software. For approval of statistical significance between groups a two-tailed t-test was performed. $P$ values < 0.05 were considered to indicate significant differences. For comparison of functional neuronal network development, slopes of linear regressions were calculated with GraphPad Prism 4 and p- and F-values were given.

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#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions to the manuscript

MD and JW conceived and designed the manuscript. MD supported the study financially. HPH supported the study administratively. JW, SDH, SI and JMW collected and assembled the data. JW and MD wrote the manuscript. The manuscript was finally approved by MD, HPH and CRR. CRR conceived and afforded the patch clamp recordings. SDH performed and analysed the patch clamp recordings. SI performed the MEA recordings.
All authors read and approved the final manuscript.

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- experimental design 80%
- experiments 80%
- figures: 90%
- manuscript writing: 80%

## 2.2 Interferon gamma and sonic hedgehog signaling are required to dysregulate murine neural stem/precursor cells

On the following pages (28-34) the research manuscript “Interferon gamma and sonic hedgehog signaling are required to dysregulate murine neural stem/precursor cells” is presented, as published in the journal PLoS One.

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# Interferon Gamma and Sonic Hedgehog Signaling Are Required to Dysregulate Murine Neural Stem/Precursor Cells

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## Abstract

***Background:*** The pro-inflammatory cytokine interferon gamma (IFNγ), a key player in various neurological diseases, was recently shown to induce a dysregulated phenotype in neural stem/precursor cells (NSPCs) that is characterized by the simultaneous expression of glial and neuronal markers and irregular electrophysiological properties. Thus far, the mechanisms of this phenomenon have remained unclear.

***Methodology/Principal Findings:*** To determine if binding of the signal transducers and activators of transcription (Stat 1) to the sonic hedgehog (SHH) promoter is important for this phenomenon to occur, chromatin immunoprecipitation and pharmacological inhibition studies were performed. We report here that the activation of both the Stat 1 and SHH pathways is necessary to elicit the dysregulated phenotype.

***Conclusions/Significance:*** Thus, blocking these pathways might preserve functional differentiation of NSPCs under inflammatory conditions leading to more effective regeneration.

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## Introduction

The pro-inflammatory cytokine IFNγ is mainly produced by cytotoxic $CD8^+$ T-cells, natural killer cells [1], astrocytes, fibroblasts and endothelial cells [2,3,4] under normal or pathological conditions after stroke, cerebral traumata or in the course of inflammatory brain diseases [5]. As previously reported, IFNγ affects murine NSPCs *in vitro* leading to a dysregulated phenotype [6]. This phenotype is characterized by reduced proliferative activity and a synchronous up-regulation of mature neuronal and glial markers also in the presence of growth factors. The IFNγ-induced phenotype bears electrophysiological properties that are indiscernible from undifferentiated NSPCs. The mechanisms involved in IFNγ-induced NSPC dysregulation are unknown. Up-regulation of Stat 1 after IFNγ exposure suggested one of the common down-stream pathways of IFNγ to be involved in NSPC dysregulation. Interestingly, also SHH was considerably up-regulated pointing to a possible crosstalk of IFNγ signaling and SHH production during formation of the dysregulated NSPC phenotype. Similar mechanisms were observed during the differentiation of granular neuron precursor cells of postnatal mice [7] or primary mouse and human pre-adipocytes [8] under IFNγ influence.

## Results

### Genotypic and Phenotypic Dysregulation of NSPCs and Effects of SHH Antagonism

To verify if SHH signaling is involved in generating the IFNγ-induced phenotype in NSPCs, we antagonized SHH signaling with cyclopamine during IFNγ exposure. Cyclopamine is known to inhibit SHH signaling due to binding, inactivation and change in protein conformation of smoothened [9]. Smoothened is a seven-pass membrane protein and G Protein coupled receptor that regulates the translocation of Gli transcription factor to the nucleus [9]. In a first set of experiments, we verified the induction of the dysregulated $GFAP^+/\beta III\text{-tubulin}^+$ phenotype by IFNγ treatment of NSPCs under the influence of growth factors. As previously reported, we could reliably induce the $GFAP^+/\beta III\text{-tubulin}^+$ phenotype by 1000 U/ml IFNγ (Figure 1a). Also, on mRNA level we demonstrated an up-regulation of both, GFAP and βIII-tubulin after IFNγ exposure (Figure 1b). We then inhibited the SHH pathway during IFNγ-induced dysregulation. For this purpose, we simultaneously applied cyclopamine and IFNγ. And indeed, cyclopamine nearly completely prevented the generation of $GFAP^+/\beta III\text{-tubulin}^+$ cells. These findings were confirmed on protein and on mRNA level by means of immunocytochemistry and real-time quantitative PCR (Figure 1a+b). To investigate effects of SHH antagonism on proliferating, non-dysregulated NSPCs, we also applied cyclopamine without IFNγ. We found no

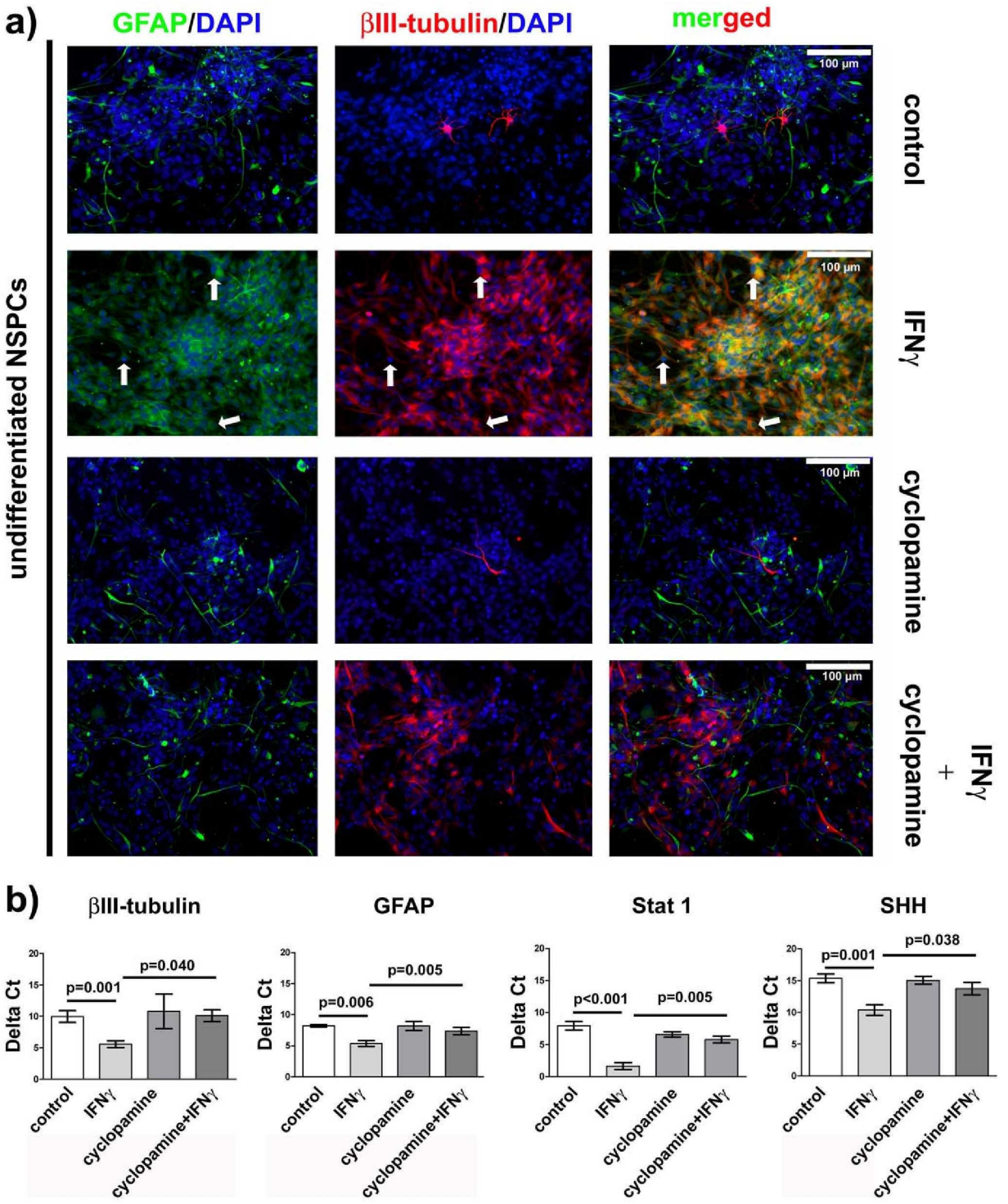


**Figure 1. Effects of IFNγ in NSPCs are blocked by cyclopamine.** In Figure a: Photomicrographs of undifferentiated murine NSPCs are given. The left panel shows an immunocytochemical staining for GFAP, the middle panel for βIII-tubulin and the right panel shows both. The first row represents the control, and the second row represents the IFNγ-treated cells. The third row shows cells that are treated with cyclopamine alone, and the fourth row shows cells treated with both substances. In Figure b: The results of the real-time quantitative PCR experiments for GFAP, βIII-tubulin, Stat 1 and SHH gene expression are depicted. Delta CT values are means +/− standard error of mean (SEM). Independent experiments were repeated for three times in triplicate.
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significant differences in the expression of βIII-tubulin or GFAP in the non-treated control or the cyclopamine-treated group (Figure 1a+b).

We monitored the expression levels of Stat 1 and SHH in all 4 experimental groups by real-time quantitative PCR, since we postulate a crosstalk of IFNγ signaling and SHH pathway, probably mediated by phosphorylated Stat 1 leading to the establishment of the dysregulated phenotype of NSPCs. We found Stat 1 and SHH to be up-regulated after IFNγ exposure in comparison to the control group. Cyclopamine inhibited this IFNγ-induced up-regulation and no significant changes in Stat 1 and SHH expression in comparison to control were observed when cultures were treated with cyclopamine alone (Figure 1b).

## Gen-expression Levels of SHH and Stat 1 and Population Size of NSPCs Correlate to the Concentration of IFNγ

To detect a possible concentration threshold from where IFNγ induces SHH and/or Stat 1 up-regulation, we performed experiments with different concentrations of IFNγ. We found a significant up-regulation of SHH and Stat 1 at an IFNγ concentration of 100 Units per ml and higher (Figure 2 a+b). We then investigated the population size of undifferentiated NSPCs under the influence of different concentrations of IFNγ since we speculate, that the above mentioned concentrations of 100 Units per ml or higher will also influence their proliferation. Undifferentiated NSPC populations treated for 72 hours with the indicated concentrations of IFNγ showed a significant decrease in optical density, which represents the measuring value in the MTT assay indicative for a reduced population size due to reduced proliferation or induced apoptosis. As the MTT assay measures metabolic activity, a bias towards lower values in differentiated cultures after IFNγ-treatment cannot completely be excluded. Again, these findings were significant with concentrations of 100 Units per ml or higher (Figure 2c).

## Administration of Recombinant SHH alone does not Lead to a Dysregulated Phenotype in NSPCs

As SHH antagonism diminished the generation of IFNγ-induced $GFAP^+/\beta III\text{-}tubulin^+$ phenotypes, we investigated if SHH alone is sufficient to induce this phenotype which would implicate a linear down-stream signaling IFNγ – SHH – $GFAP^+/\beta III\text{-}tubulin^+$ phenotype. For this purpose, we applied 300 ng/ml recombinant murine SHH to undifferentiated NSPCs under the influence of growth factors. However, neither an increase in numbers of $GFAP^+/\beta III\text{-}tubulin^-$ or $GFAP^{-}/\beta III\text{-}tubulin^+$ cells nor the appearance of dysregulated $GFAP^+/\beta III\text{-}tubulin^+$ cells was detectable. These findings were confirmed by immunocytochemistry and on gene expression level by real-time quantitative PCR (Figure 3a+b). Also, no alteration in Stat 1 gene-expression was detectable after SHH treatment. Instead, we found SHH gene-expression to be significantly up-regulated after administration of recombinant SHH to undifferentiated NSPCs implying a positive autoregulatory loop. These results suggest, that the dysregulated phenotype of NSPCs is not inducible by SHH alone, even when applied at higher concentrations.

## Stat 1 Interaction with the SHH Promoter

To further investigate the up-regulation of SHH after IFNγ treatment and to clarify if Stat 1 binds to an IFNγ-activated side (GAS) sequence in the promoter region of the SHH promoter, we performed chromatin immunoprecipitation. For this purpose NSPCs were treated with IFNγ or cultured under control conditions and afterwards chromatin immunoprecipitation (with

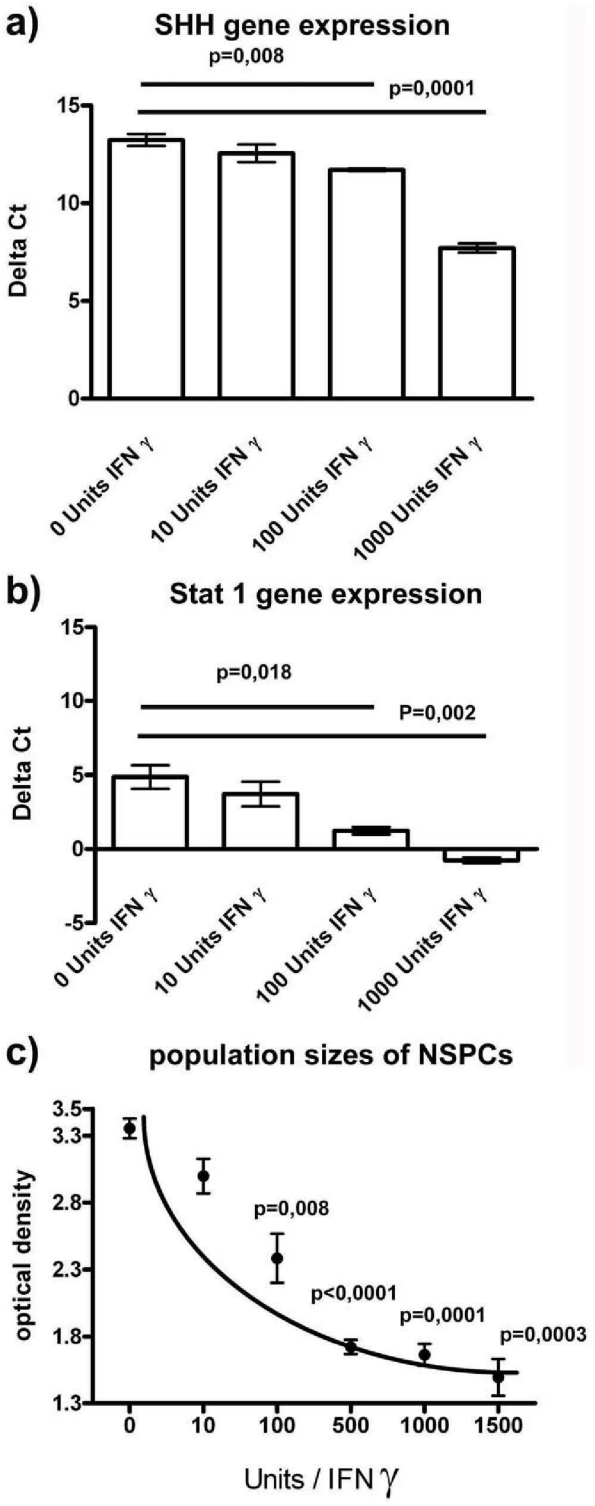


**Figure 2. The effects of IFNγ treatment in NSPCs are dose-dependent.** In Figure 2 a, b, c: Gene expression of SHH and Stat 1 or the population sizes of the NSPCs are shown in relation to illustrated concentrations of IFNγ. Values are means +/− standard error of mean (SEM). Independent experiments were repeated for three times in

triplicate. For concentration dependent gene expression and proliferation studies, NSPCs were seeded at an equal density at day 0, than incubated with different amounts of IFNγ as indicated in the figure. The experimental read out took place three days after the start of the experiment.
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Stat 1 antibody, normal rabbit IgG antibody or Histon H3 antibody as positive control) was performed. After immunoprecipitation, real-time quantitative PCR against a GAS Sequence in the SHH promoter and a reference gene sequence was used to analyze possible binding of Stat 1 in the SHH promoter region. We chose primer sequences that were specifically designed to amplify GAS sequences in the SHH promoter [7]. We could show a 1.5 fold increase in the chromatin immunoprecipitation sample of IFNγ treated cells for the SHH promoter. Our findings suggest, that a phosphorylated dimer of Stat 1 is able to enter the nucleus, and then binds to a GAS sequence in the SHH promoter (Figure 4a) and therefore leads to the up-regulation of SHH after IFNγ treatment. A possible mechanism for this interaction is visualized in Figure 4b.

## Discussion

In the last few years various studies were aimed at elucidating the role of the pro- inflammatory cytokine IFNγ during neurodegenerative or neuroinflammatory conditions and its impact on NSPCs. In those reports Janus-faced properties of IFNγ were described. On the one hand, pro neurogenic effects of IFNγ on neural stem cell differentiation were described [10,11,12,13,14]. On the other, a number of publications report a negative effect on neuronal differentiation and neurite outgrowth [15,16,17,18,19,20,21]. A dualistic role of IFNγ was also seen when proliferation and differentiation of astrocytes was investigated [5,22,23].

We recently reported that IFNγ leads to phenotypic and genotypic dysregulation in a substantial portion of murine E14 neurosphere-derived stem/precursor cells. This dysregulation is characterized by a simultaneous expression of neuronal and glial markers in immature NSPCs as soon as 3 to 6 hours after application of IFNγ. Furthermore, it was shown that IFNγ causes an up-regulation of SHH and Stat 1 mRNA and a decrease in the population size.

We now extended those investigations and found that the IFNγ-induced decrease in the population size is concentration dependent beginning with 100 Units per ml. Although there might be a

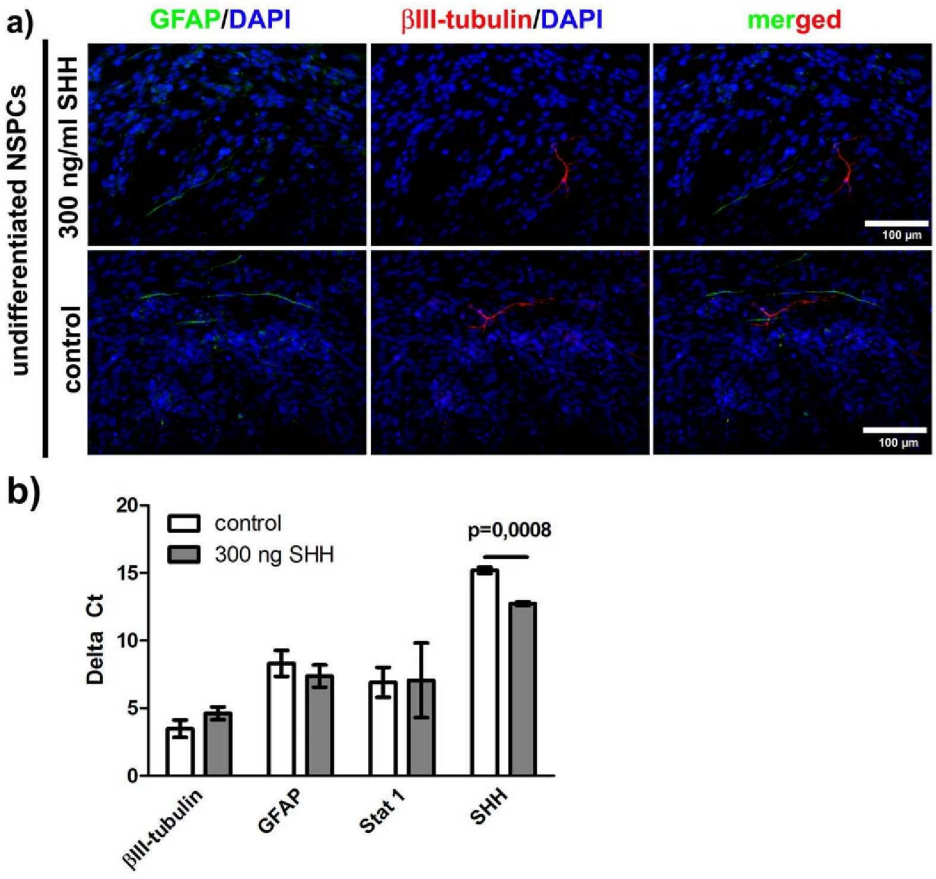


**Figure 3. The administration of recombinant SHH does not lead to a dysregulated phenotype in NSPCs.** In Figure 3a: Immunocytochemical photomicrographs of undifferentiated murine NSPCs are presented under the illustrated conditions. In Figure 3b: The results of real-time quantitative PCR are depicted. The values represent delta CT values. Delta CT values are means +/− standard error of mean (SEM). Independent experiments were repeated for three times in triplicate.
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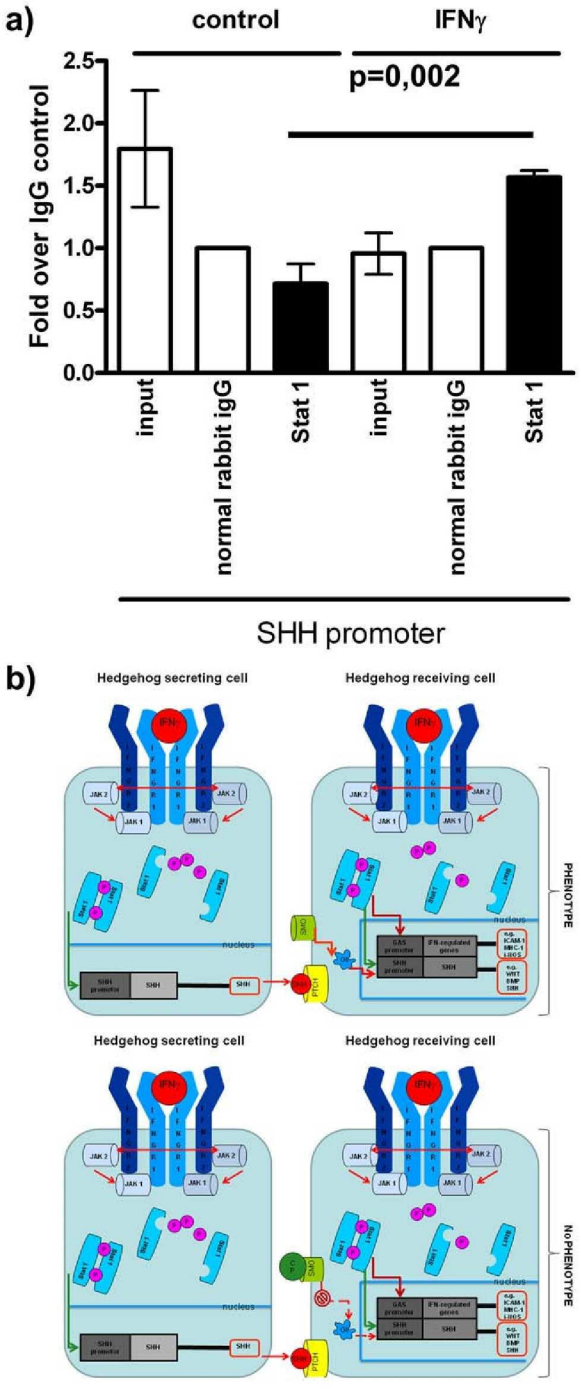


**Figure 4. The mechanism of IFNγ-mediated dysregulation of NSPCs.** In Figure 4a: The results of the chromatin immunoprecipitation of the SHH promoter region with a Stat 1 antibody are shown. In Figure 4b: A schematic drawing of potential pathways that might be involved in IFNγ-induced NSPC dysregulation. CP: Cyclopamine, SMO: smoothened, PTCH: patched, GAS: interferon-*gamma-activated site* (GAS), ICAM-1: intercellular adhesion molecule 1, MHC-1: major histocompatibility complex 1, i-NOS: inducible nitric oxide synthase, JAK2: janus kinase 2, Stat 1: signal transducers and activators of transcription 1.
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bias towards lower population sizes in differentiated cultures that bear a lower metabolic activity, the IFNγ-induced reduced values in the MTT assay are best explained by anti-proliferative and/or pro-apoptotic effects. This is substantiated by the observation that the IFNγ-induced dysregulation was accompanied by an up-regulation of caspase 3/7 and a decrease in BrdU uptake, suggesting IFNγ-related anti-proliferative effects and enhanced apoptosis [6].

We found that also the IFNγ-induced up-regulation of SHH and Stat 1 is concentration dependent beginning with 100 Units per ml. We speculated that SHH, as one of the major morphogenes in brain development, is involved in the formation of $GFAP^{+}/\beta III\text{-tubulin}^{+}$ cells and that Stat 1 might be involved in SHH up-regulation. To substantiate this hypothesis, we first investigated if Stat 1 can bind to the SHH promoter. For this, we performed a chromatin immunoprecipitation assay and confirmed that a phosphorylated dimer of Stat 1 is capable to enter the nucleus in order to bind to a GAS sequence in the SHH promoter region and therefore leads to an up-regulation of SHH. This shows that IFNγ-related Stat 1 up-regulation is directly linked to the induced SHH pathway since its known, that the phosphorylated dimers of Stat 1 enter the nucleus and function as transcription factors [24,25,26].

To investigate if an activated SHH pathway is directly involved in generating the IFNγ-induced phenotype, we antagonized the SHH pathway by administration of cyclopamine. Cyclopamine is known to inhibit the activity and down-stream-signaling of smoothened by a binding-induced conformational change in the smoothened protein [9]. We were able to show that the IFNγ-induced dysregulation of NSPCs is significantly ameliorated after antagonizing SHH signaling indicating that SHH signaling is necessary to induce the dysregulated phenotype. This finding was confirmed by immunocytochemistry and real-time quantitative PCR. Notably, we were not able to completely block IFNγ-induced NSPC dysregulation by cyclopamine. This could probably be due to the low concentration of cyclopamine as it was used in our study to prevent toxicity. After identification of SHH as mediator for NSPC dysregulation, we added recombinant murine SHH in high doses to undifferentiated NSPCs, to clarify if the dysregulated phenotype can be induced solely via SHH signaling downstream from IFNγ. However, SHH alone was not able to induce NSPC dysregulation. This indicates that the IFNγ-induced dysregulated phenotype is depending on activation of the SHH pathway and simultaneously on other IFNγ-related effects. Thus, the dysregulated phenotype could either be induced via a simultaneous SHH- and Stat 1-activation of the SHH promoter or, for instance, via simultaneous SHH-activation of the SHH promoter and activation of common [27,28,29] IFN-regulated genes (IRGs) or IFN-stimulated genes (ISGs) by the JAK/Stat cytokine pathway. We summarized this in the schematic drawing. The IFNγ-activated site (GAS) element in the SHH promoter region might therefore be involved in SHH up-regulation or in a direct co-activation of the dysregulated phenotype.

The binding of phosphorylated Stat 1 to the SHH promoter after IFNγ exposure was previously shown by Sun et al. [7]. Interestingly, Sun and colleagues did not find a dysregulated phenotype or a reduced proliferation, but they described a clear up-regulation of SHH after IFNγ administration and they also describe a binding of Stat 1 to the SHH-promoter region. Notably, the cells used by Sun and colleagues were granular neural precursor cells from postnatal mice, in contrast to NSPCs or the murine ES cell-derived neural stem cells used in our study. This suggests that effects mediated by IFNγ exposure seem not only to depend on the examined brain region but also on the develop-

mental stage and the cell type investigated. It is even more surprising that Todoric and colleagues described a cross-talk of IFNγ and SHH in human and murine pre-adipocytes as well [8]. This leads to the hypothesis, that crosstalk of IFNγ and SHH is an important genetically conserved factor in cellular response to inflammatory signals, since this pathway is not limited to the murine species or a special cell type. Further experiments to elucidate this hypothesis could be I) the application of JAK inhibitors, to block IFNγ signaling to the nucleus and studying the SHH expression afterwards, II) the administration of exogenous SHH together with IFNγ, to see if the amount of dysregulated cells increases by exogenously applied SHH, as well as III) a Stat 1 phosphorylation assay to measure the increase of phosphorylated Stat 1 molecules in a dependency to the used Units of IFNγ. This experiment would further confirm a direct receptor mediated phosphorylation of Stat 1 in NSPCs.

Taken together these findings provide new evidence for the importance of pro-inflammatory signals in cell fade- and differentiation-decisions since SHH is an important morphogene in brain development and the neural stem cells niche [30].

Therefore, we claim a complex and diverse role of IFNγ as mediator of dysregulation in NSPCs.

## Materials and Methods

### Neurosphere Cultures

Neurospheres were generated from fourteen-day-old wild type C57BL/6J mouse embryos. Cell preparation and animal care were performed in compliance with the German Animal Protection law (State Office, Environmental and Consumer Protection of North Rhine-Westphalia). Ganglionic eminences were removed, mechanically dissociated and seeded in DMEM/F12 culture medium (1:1; Invitrogen, Karlsruhe, Germany) containing 0.6% Glucose (Sigma-Aldrich, Hamburg, Germany), glutamine (2 mM; Invitrogen), sodium bicarbonate (3 mM; Invitrogen), Hepes buffer (5 mM; Invitrogen) and B27 (20 µl per ml; Invitrogen). For generation and expansion of neurosphere cells, epidermal growth factor (EGF) (Tebu-bio, Le Perray en Yvelines Cedex, France) and basic fibroblast growth factor-2 (FGF-2) (Tebu-bio) were added to a final concentration of 20 ng per ml each.

### IFNγ Treatment and Immunocytochemistry

For immunocytochemistry, neurosphere cells were dissociated to a single cell suspension and plated on poly-L-ornithine (PLO; 0.001%; Sigma-Aldrich) and fibronectin (5 µg/ml; Tebu-bio) coated cover slips (VWR International, Darmstadt, Germany) at a density of 125,000 cells per $cm^2$. After 3 days under the influence of EGF and FGF-2 (20 ng/ml both Tebu-bio), cells were assigned to the different experimental groups. To verify the marker expression of undifferentiated (proliferating) neural populations under control or IFNγ treated conditions, cultures were kept under the influence of EGF/FGF-2 without or with IFNγ (1000 U/ml; Millipore) until fixation for further 3 days (NSPC-p -IFNγ/+IFNγ). For control experiments, only phosphate-buffered saline solution (PBS; 1X; Invitrogen) was added to the medium. Primary antibodies used at 4°C overnight were the monoclonal mouse antibody against βIII-tubulin (Tuj1; 1:500; R&D Systems, Minneapolis, USA or 1:800, Abcam, Cambridge, UK) and the polyclonal rabbit antibody against glial fibrillaric acid protein (GFAP) (1:500; Dako, Hamburg, Germany or 1:1000; Abcam). For detection of primary antibodies, fluoresceine-isothiocyanate (FITC; 1:500; Millipore) and indocarbocyanine (Cy3; 1:800; or Cy5; 1:200; Millipore) coupled secondary antibodies were used. For negative controls, primary antibodies were omitted in each experiment. To measure the total population of cells, Dapi positive cell nuclei were counted. On every cover slip, at least 100 cells were counted.

### MTT-Assay

To analyze the population size of NSPCs, the optical density, indicative of conversion of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) into formazan crystals which takes place in live cells only, was determined after IFNγ treatment at indicated concentrations. An OD value of 0.5 represents approximately 50,000, and an OD value of 1.0 represents approximately 100,000 live NSPCs. The population size was measured after 72 hours of IFNγ treatment. Therefore cells were incubated with MTT (final concentration 0.5 mg/ml) and media one half each and incubated for 3–5 hours at 37°C afterwards the supernatant was discarded and a lysis with DMSO was performed.

### Real-time Quantitative PCR

RNeasy Kit (Qiagen) was used for RNA isolation of cultured NSPCs. Then a reverse transcription into cDNA (ABI, Darmstadt, Germany) was performed. Real-time quantitative PCR was carried out by the usage of the 7500 fast or 7500 real-time quantitative PCR cycler (ABI, Darmstadt, Germany). Either SYBR green master mix (Qiagen) or equivalent chemistry from another supplier (Quantace, London, UK) was used. The specific primers for genes of interest or the housekeeping gene (glyceraldehyde- 3-phosphate dehydrogenase, GAPDH) were either purchased (QuantiTect primer assays, Qiagen) or self designed (BioTEZ, Berlin, Germany). The genes of interest (target gene) in IFNγ-treated groups or control groups (PBS-treated) were analyzed in at least 3 independent cultures in triplicate each. Every experiment in IFNγ-treated or control (PBS-treated) groups provided delta CT values (ΔCT: gene of interest minus reference gene). NSPCs were cultivated for 72 hours with IFNγ at the indicated concentrations before the RNA was harvested.

### Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (CHIP-Assay) was performed by using the simpeCHIP IP Kit (cell signaling) according to the manufacturers instruction, with chip grade antibodies against Stat 1 (cell signaling). Briefly, NSPCs were seeded on PDL and fibronectin coated dished and treated with IFNγ or PBS as control for three days and then fixed with 1% PFA for 10 minutes, harvested and lysed. Chromatin was digested by sonification and incubation with micrococcal nuclease. For chromatin immunoprecipitation the isolated chromatin was incubated with normal rabbit IgG antibody as negative control, Histon H3 antibody as positive control and Stat 1 antibody over night at 4°C. Afterwards the chromatin/antibody mixture was incubated with protein G agarose beads for 2 hours at 4°C. The chromatin was washed and eluted from the protein g agarose beads, the DNA was reverse cross linked to single strand formation and purified over silica membranes. After purification, the isolated DNA was used for real-time quantitative PCR using primers against the SHH promoter regions and the histon H3 complex.

### Statistical Analyses

Experiments were repeated with independent cultures at least three times in triplicate each. The resulting data sets were statistically analyzed und illustrated using the GraphPad Prism 4 (GraphPad Software Inc., San Diego,CA, USA, 2003) software.

For approval of statistical significance between groups, a two-tailed unpaired t-test was performed. P values $<0.05$ were considered to indicate significant differences.

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## Author Contributions

Conceived and designed the experiments: JW MD. Performed the experiments: JW. Analyzed the data: JW MD. Contributed reagents/materials/analysis tools: JW MD HPH. Wrote the paper: JW MD.

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- manuscript writing: 80%

## 2.1 Species-dependent differences of embryonic stem cell-derived neural stem cells after Interferon gamma treatment

On the following pages (37-43) the research manuscript “Species-dependent differences of embryonic stem cell-derived neural stem cells after Interferon gamma treatment” is presented, as published in the journal Frontiers in Cellular Neuroscience.

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ORIGINAL RESEARCH ARTICLE
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# Species-dependent differences of embryonic stem cell-derived neural stem cells after Interferon gamma treatment

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Pluripotent stem cell (pSC)-derived, neural stem cells (NSCs) are actually extensively explored in the field of neuroregeneration and to clarify disease mechanisms or model neurological diseases *in vitro*. Regarding the latter, proliferation and differentiation of pSC-derived NSCs are investigated under the influence of a variety of different substances among them key players of inflammation. However, results generated on a murine genetic background are not always representative for the human situation which increasingly leads to the application of human cell culture systems derived from human pSCs. We investigated here, if the recently described interferon gamma (IFNγ)-induced dysregulated neural phenotype characterized by simultaneous expression of glial and neuronal markers on murine NSCs (Walter et al., 2011, 2012) can also be found on a human genetic background. For this purpose, we performed experiments with human embryonic stem cell-derived NSCs. We could show that the IFNγ-induced dysregulated neural phenotype cannot be induced in human NSCs. This difference occurs, although typical genes like signal transducers and activators of transcription 1 (Stat 1) or interferon regulatory factor 9 (IRF-9) are similarly regulated by IFNγ in both, murine and human populations. These results illustrate that fundamental differences between murine and human neural populations exist *in vitro*, independent of anatomical system-related properties.

**Keywords: human neural stem cells, inflammation, interferon-gamma, neurodegeneration, IFNγ**

## INTRODUCTION

Neural stem cells (NSCs) are multipotent and can give rise to the three principle cell types found in the adult mammalian brain, namely neurons, astrocytes, and oligodendrocytes. *In vivo*, they can be found in the hippocampus and subventricular zone where they mainly give rise to new neurons in the dentate gyrus and olfactory bulb, a process called neurogenesis (Kempermann, 2002, 2011). *In vitro*, they can be kept under proliferative conditions exhibiting immature neural markers like nestin and Sox2 due to the stimulus of growth factors, or differentiate to the above mentioned mature cell types when growth factors are withdrawn (Temple and Qian, 1996; Temple, 2001). Various regenerative approaches are aimed at using the capacity of NSCs as a therapeutic agent by transplanting them into injured brain regions (Barker et al., 2003; Harrower and Barker, 2005; Gogel et al., 2011). Additionally, NSCs from various sources are increasingly used to simulate diseases *in vitro* in order to set up model systems that can easily be manipulated and investigated. A specific and important advantage of *in vitro* model systems for neurological diseases is the possibility to use a human genetic background on the basis of human pluripotent stem cells (pSCs) in order to exclude, for instance, murine-specific phenomena. One interesting question, that could be clarified *in vitro* under controlled conditions, is how inflammatory processes that consistently occur in various neurological diseases act on NSCs. As *in vivo* neurogenesis is more and more accepted to contribute to processes like, for instance, memory function (Deng et al., 2009, 2010), it appears to be of interest to characterize effects of pathological processes such as inflammation on NSCs. Inflammation itself may lead to damage in brain tissue and represents either a primary disease entity or a secondary phenomenon following (Campbell, 2005), for instance, cerebral ischemia (Whitney et al., 2009). Interferon gamma (IFNγ), a pro-inflammatory key player, is a cytokine that is secreted by various cell types such as cytotoxic $CD8^+$ T-cells, natural killer cells (Griffin, 1997), astrocytes, fibroblasts, and endothelial cells (Rady et al., 1995; De Simone et al., 1998; Wei et al., 2000). IFNγ signaling takes place via the IFNγ receptor which consists of two chains, situated in the cell membrane with an extra- and intracellular part (Schreiber and Farrar, 1993). The structure and the amino acid sequence of the murine and the human IFNγ protein and its receptor differ, although the physiological function remains the same (Farrar and Schreiber, 1993). These structural differences are leading to species-specific IFNγ—IFNγ receptor interactions with human IFNγ affecting only human and other primate cell types and vice versa (Schreiber et al., 1992; Schroder et al., 2004). IFNγ receptors were found on murine NSCs and therefore, effects of IFNγ on murine NSCs and related alterations in neurogenesis *in vivo* (Kim et al., 2002; Lin et al., 2004; Sweeten et al., 2004; Wang et al., 2004, 2008) and *in vitro* (Kim et al., 2007; Makela et al., 2010) were excessively

explored. However, only little is known about the response of human NSCs (hNSCs) to IFNγ. We previously investigated effects of IFNγ on murine embryonic day 14-derived stem/precursor cells (msNSPCs) and murine embryonic stem cell-derived NSCs *in vitro* (Walter et al., 2011, 2012) (both populations are referred to as mNSCs in the following text). Predominantly in proliferative mNSC cultures, we found that IFNγ leads to a dysregulated phenotype, characterized by synchronous expression of neuronal and glial markers despite the presence of growth factors. This was accompanied by an unusual electrophysiological phenotype on single cell level preventing the ability to form synchronously bursting functional neuronal networks after differentiation. We also demonstrated an IFNγ-related significant up-regulation of sonic hedgehog (SHH) and Stat 1, key down-stream signals that are important for induction of the above mentioned phenotype (Walter et al., 2012). To assess the relevance of these findings with respect to the human situation, we (1) treated human embryonic stem cell-derived hNSCs with this pro-inflammatory cytokine and (2) measured IFNγ concentrations in cerebrospinal fluid (CSF) specimens collected from patients suffering from different nervous system diseases.

## RESULTS

### hNSCs EXPRESS IFNγ RECEPTOR I AND II

In a first step, we immunocytochemically characterized the hNSC population generated from immature pluripotent embryonic stem cells. After neural pre-differentiation, almost all cells expressed nestin and most cells (>80%) expressed both, nestin and Sox2 (**Figure 1A**). After withdrawal of bFGF, NSCs terminally differentiated into βIII-tubulin$^+$ neurons or GFAP$^+$ astrocytes (**Figure 1A**). As the expression of membrane-bound IFNγ receptors (2 IFNγ receptor-1 chains and 2 IFNγ receptor-2 chains) is necessary to transduce the IFNγ signal, we performed immunocytochemical labelings against both receptor chains and were indeed able to demonstrate their expression (**Figure 1B**). We further investigated the expression levels of both receptor chains on mRNA level by means of real-time quantitative PCR. We compared these data with those generated on a murine background and found, that murine and human NSCs did not show significant differences (**Figure 1C**). Data were generated with the Wicell H9 line. We also confirmed these findings by using the HUES 6 line (data not shown).

### hNSCs DO NOT EXPRESS THE DYSREGULATED PHENOTYPE AFTER IFNγ EXPOSURE

One major characteristic of the IFNγ-induced mNSC dysregulation is the coexpression of neuronal and glial markers under the influence of growth factors that normally hold NSCs in an immature and proliferating state (**Figure 2A**). This phenomenon is visible after a 3-days treatment with 1000 U/ml IFNγ and leads to a portion of around 39% of all cells that co-express GFAP and βIII-tubulin. The detailed characterization of this phenomenon is published elsewhere (Walter et al., 2011). However, when immature and proliferating hNSC populations under the influence of growth factors were treated with human recombinant IFNγ in identical concentrations compared to the murine situation, we were not able to detect this phenomenon (**Figure 2A**). These

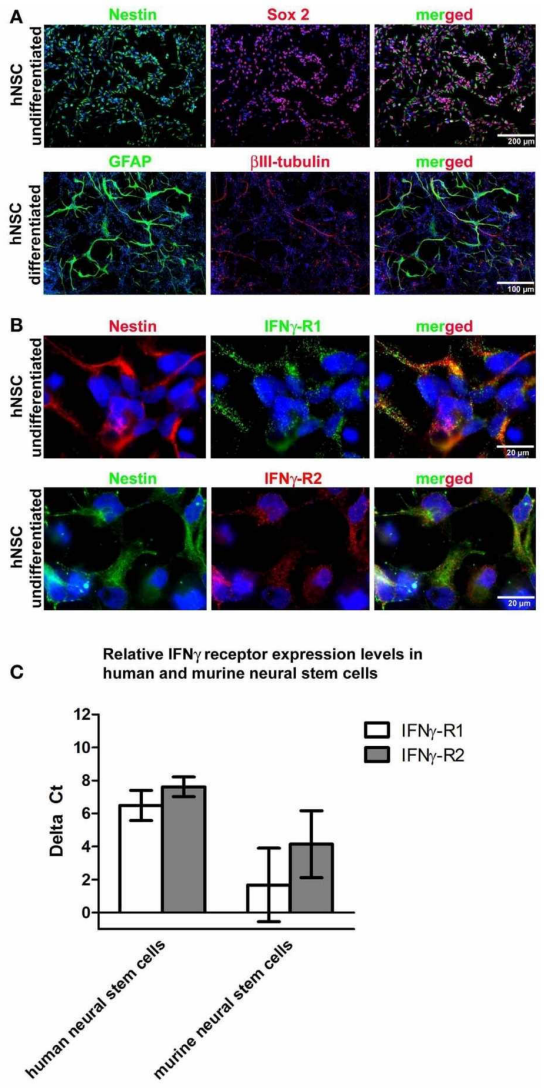


**FIGURE 1 | hNSC express IFNγ-RI and IFNγ-RII.** In **(A)** representative photomicrographs of undifferentiated and differentiated hNSCs (Wicell h9 line) are given. To characterize the undifferentiated stage, proliferating cells under the influence of growth factors were seeded and then immediately PFA fixed for immunocytochemistry. To characterize the differentiated stage, cells were seeded and differentiated for four weeks with an initial stepwise reduction of growth factors. In the upper panel immunocytochemical stainings against the neural precursor markers nestin and Sox2 in undifferentiated hNSCs are given demonstrating that the pre-differentiation protocol which starts with pluripotent stem cells yields populations highly enriched in neural precursor cells. In the lower panel immunocytochemical stainings against βIII-tubulin and GFAP in differentiated hNSCs are given demonstrating the terminal differentiation of hNSCs into neurons and astrocytes. In **(B)** representative photomicrographs of undifferentiated hNSCs (Wicell h9 line) are given. A co-immunocytochemical staining against nestin and IFNγ-RI is given in the upper panel and against nestin and IFNγ-RII in the lower panel showing that both IFNγ receptor subunits are expressed in undifferentiated hNSCs. In **(C)** gene expression levels of IFNγ-RI and IFNγ-RII in undifferentiated hNSCs (Wicell h9 line) and mNSCs is shown.

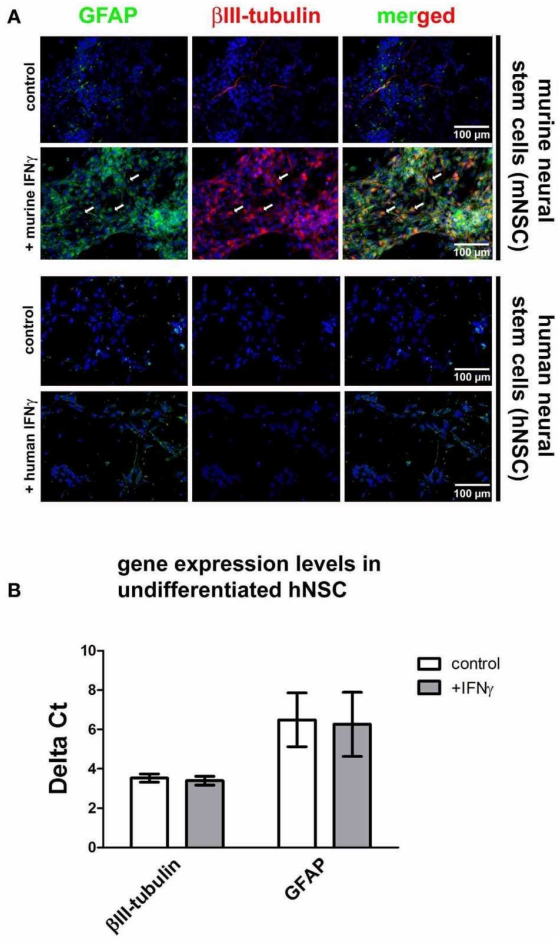


**FIGURE 2 | Effects of IFNγ on hNSCs and mNSCs.** In **(A)** photomicrographs of undifferentiated hNSCs (Wicell h9 line) and mNSCs are given. A βIII-tubulin and GFAP co-staining under control conditions and after IFNγ treatment is shown. The upper to panels show mNSCs, the lower two panels show hNSCs. In mNSCs, IFNγ typically leads to a strong up-regulation of neuronal (βIII-tubulin) and glial (GFAP) markers with many cells simultaneously expressing both markers (arrows and the merged picture). In hNSCs no IFNγ-induced up-regulation of these markers was detected. In **(B)** gene expression levels of βIII-tubulin, GFAP, and SHH in undifferentiated hNSCs (Wicell h9 line) either under control conditions or after IFNγ treatment are shown. The results demonstrate that there is, also on mRNA level, no IFNγ-induced up-regulation of cell type-specific markers.

results were also confirmed on mRNA level (**Figure 2B**). Data were generated with the Wicell H9 line. We also confirmed these findings by using the HUES 6 line (data not shown).

### IFNγ-REGULATED GENES IN hNSCs AND EFFECTS OF IFNγ ON THE POPULATION SIZE OF hNSCs

As previously reported, the genes for inducible nitric oxid synthase (i-NOS) (Komatsu et al., 1996), IRF-9 (Ousman et al., 2005), c-Myc (Ramana et al., 2000), major histocompatibility complex 1 (MHC 1) (Johansson et al., 2008) and Stat 1 (Lehtonen et al., 1997) are significantly regulated in murine cells after IFNγ exposure. We now investigated the expression of these down-stream signaling genes after IFNγ exposure in hNSCs by means of real-time quantitative PCR. We found all of these genes to be significantly up-regulated after IFNγ exposure also in hNSCs (**Figure 3A**). As previously reported, we also found SHH to be significantly up-regulated in mNSCs after IFNγ treatment. Interestingly, we could not detect that phenomenon in hNSCs. Another IFNγ-induced phenomenon on a murine background was a strong reduction of the population size of undifferentiated mNSCs. However, we were not able to detect a significant decrease in the population size of hNSCs after IFNγ exposure (**Figure 3B**). Data were generated with the Wicell H9 line. We also confirmed these findings by using the HUES 6 line (data not shown).

### IFNγ IS UP-REGULATED IN THE CEREBROSPINAL FLUID OF PATIENTS SUFFERING FROM DIFFERENT DISEASES

In contrast to the extensively described up-regulation of IFNγ in animal models of human central nervous system diseases, there is only very limited information about amounts of IFNγ in human individuals suffering from neurological diseases. Therefore, we measured the concentration of IFNγ in cerebrospinal fluid (CSF) specimens, taken after lumbar punction from patients suffering from multiple sclerosis, peripheral nervous plexus affections, Alzheimer dementia, viral myelitis, or stroke. We found, that patients suffering from multiple sclerosis during an acute relapse showed a significant increase of IFNγ (three control samples vs. three MS samples). Due to the limitation of patient material, we could only screen single patients with other diseases (one patient per other indicated disease). But also here we found a clear trend toward an up-regulation of IFNγ in the CSF (**Figure 3C**).

## DISCUSSION

We found here that IFNγ has species-dependent effects on embryonic stem cell-derived neural populations. While this pro-inflammatory key player induces a striking dysregulation in undifferentiated murine neural stem cells (mNSCs) with an unusual coexpression of neuronal and glial markers (Walter et al., 2011, 2012), it has no such effects on human NSCs although both cell populations express appropriate IFNγ receptors and up-regulate most of the classical down-stream signals like i-NOS, IRF-9, c-Myc, MHC 1, and Stat 1, which are known to mediate important IFNγ effects (Schroder et al., 2004).

One possible explanation for this diverse reaction of murine or human NSCs toward IFNγ exposure might be subtle differences in their developmental state or brain region-specific differentiation although both populations consist of proliferating nestin- and Sox2-positive neural precursors. However, the differentiation protocols used in this study were similar for both species leading to a heterogeneous population of neural cells without preference of distinct brain-region specific phenotypes (Illes et al., 2009; Lappalainen et al., 2010; Yla-Outinen et al., 2010). To account for developmental differences, we tested additional IFNγ treatment paradigms for human NSCs including a 7-days treatment or a 5-fold IFNγ concentration but could not detect an up-regulation of neuronal or glial markers or even the appearance of cells that synchronously express these markers (data not shown).

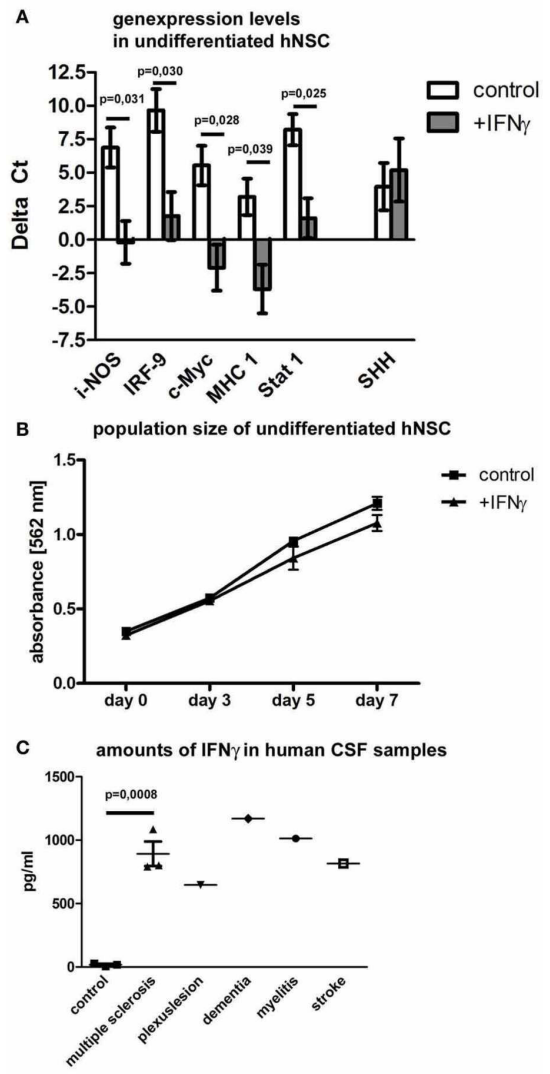


**FIGURE 3 | Down-stream gene regulation and population size of hNSCs after IFNγ exposure and *in vivo* levels of IFNγ in patients CSF samples.** In **(A)** gene expression levels in hNSCs (Wicell h9 line) of i-NOS (inducible nitric oxid synthase), IRF-9 (interferon regulatory factor 9), c-Myc, MHC1 (major histocompatibility complex 1), and Stat 1 (signal transducers and activators of transcription 1) are given either under control conditions or after IFNγ treatment. Results show that IFNγ significantly up-regulates important down-stream factors except of SHH. Values are means ± standard error of mean (SEM). Experiments were performed in triplicate and repeated independently at least three times. In **(B)** population size of hNSCs (Wicell h9 line) over 7 days is measured by means of an MTT-assay. Population sizes of two groups, hNSCs under control conditions or after IFNγ treatment, are compared. Results show that IFNγ does not affect the population size of hNSCs. Values are means ± standard error of mean (SEM). Experiments were performed in triplicate and repeated independently at least three times. In **(C)** amounts of IFNγ in clinical CSF samples are measured by ELISA in comparison to CSF samples of control patients. For statistical testing of the multiple sclerosis group, 3 control patients were compared with 3 multiple sclerosis patients [unpaired two-tailed *t*-test, value is mean ± standard error of mean (SEM)].

Another explanation for the divergent reactions after IFNγ exposure might be a qualitatively different induction of down-stream signals. While SHH is significantly up-regulated after IFNγ treatment in mNSC populations, it remains unaltered in hNSC populations. Interestingly, recently published results of our group show that the IFNγ-induced dysregulated murine phenotype depends on both, Stat 1 and SHH signaling (Walter et al., 2012). Thus, the lack of an important down-stream signal in the human situation might explain the species-dependent differences. As it is known that human gliomas and tumorigenic NSCs express SHH (Ehtesham et al., 2007) and comprise cells that simultaneously express neuronal and glial markers (Katsetos et al., 2001; Ignatova et al., 2002; Singh et al., 2003; Walton et al., 2009), this morphogene was associated with brain tumor formation and/or growth. Interestingly, in mice a link between IFNγ and SHH signaling was observed as an ectopic expression of IFNγ was shown to induce medulloblastoma formation via SHH overexpression (Lin et al., 2004). Our results in general point to the fact that, possibly fundamental differences in cytokine-induced signaling pathways between human and mouse lead to significant differences in the development of cellular phenotypes. Nevertheless, this type of fundamental differences can also be observed within a single genetic background if, for instance, merely the developmental age of a given cellular population differs. In this regard, several studies investigated the role of IFNγ in human lymphocyte activation and found that, depending on their developmental age (neonatal vs. adult); IFNγ induces divergent down-stream signaling pathways leading to significant differences in their ability to response to pathogens (Wilson et al., 1986; Marodi et al., 1994, 2001; Marodi, 2002). Discussed mechanisms for this divergent response of neonatal or adult lymphocytes to IFNγ are a less effective Stat 1 phosphorylation in neonatal cells or an up-regulation of a new class of cytokine signaling suppressors that can inhibit JAK-Stat pathway (Endo et al., 1997; Starr et al., 1997). In summary, our results admittedly reduce the relevance of an IFNγ-induced dysregulation of undifferentiated NSCs in a human genetic background. However, regarding the above mentioned considerations, it appears to be possible that human NSCs of a different developmental stage in comparison to those we used here can still react to IFNγ exposure with an even misguided initiation of differentiation programs. Thus, our results can rather be interpreted in a way that a given cellular population *in vitro*, even on a human genetic background, might overemphasize results as it not reflects the complexity of an organism. This consideration is substantiated by observations from Johansson et al. (2008). In their study, immortalized hippocampal or striatal human neural stem/progenitor cells from 12-weeks-old fetal brains showed increased neurogenesis and MHC 1 expression after IFNγ exposure during their differentiation phase without growth factors. The divergent results in comparison to our study might simply be explained by the developmental stage of the population during IFNγ exposure as we used proliferating human NSCs under the influence of growth factors. That human fetal cells are principally able to coexpress GFAP and βIII-tubulin under non-inflammatory conditions in the ventricular and subventricular zones as well as under culture

conditions was shown by Draberova et al. (2008). Probably this geno- and phenotypic rare case is only possible in a specific time frame of human fetal development and independent from inflammatory stimulation.

To further substantiate the relevance of IFNγ during human CNS diseases, we verified the up-regulation of IFNγ in the CNS. We demonstrate here that patient CSF contains elevated amounts of IFNγ in comparison to control CSF of healthy individuals. Significantly elevated IFNγ levels were found during relapses in multiple sclerosis ($n = 3$) and we also found increased levels in individual patients suffering from peripheral nervous plexus affections, Alzheimer dementia, viral myelitis, or stroke ($n = 1$, respectively). This is in accordance with the fact that human peripheral lymphocytes can secrete up to approximately 200–500 U/ml IFNγ under neuroinflammatory conditions (Hirsch et al., 1985; Chan et al., 1991). As brain ependymal cells lack tight junctions, the CSF compartment in the brain ventricles exchanges neuroactive substances with the interstitial fluid of the brain parenchyma, including neurogenic zones (Alvarez-Buylla and Lim, 2004; Shen et al., 2008; Tavazoie et al., 2008; Ming and Song, 2011; Hartung and Dihne, 2012). This might point to a possible anatomical relationship between IFNγ accumulation within the CSF and NSCs within neurogenic zones, substantiating the relevance of IFNγ effects on NSCs in humans. In summary, our results demonstrate that data collected on a murine genetic background cannot automatically be translated to the human situation and that even on a human background results might differ depending on the developmental stage of the population and their maturation.

## MATERIALS AND METHODS

### CELL CULTURE

The human NSCs used in this study were either derived from the human embryonic stem cell lines HUES 6 (hESC facility; Harvard University; Cambridge, MA, USA) or purchased in a neuroectodermal, predifferentiated form of the human embryonic stem cell line H9 (WiCell Research Institute, Madison, WI, USA/Invitrogen, Karlsruhe, Germany). The detailed neuronal differentiation protocol is described elsewhere (Dhara et al., 2008). Use of hESCs for derivation of neural progenitors was approved by the regulatory authorities at the Robert Koch Institute, Berlin, Germany, and derivation of neural progenitors was performed as follows: the hESCs were cultivated in knockout serum replacement (KSR) media (Cowan et al., 2004) on gelatin coated plates and a feeder layer of γ-irradiated mouse embryonic fibroblasts. Neural differentiation was performed by a protocol previously described by Lappalainen et al. (2010). Briefly, hESCs were dislodged and separated from the feeder cells and afterwards dissected into clusters that contained in between 1000 and 3500 cells. These clusters were placed in neural differentiation/proliferation media that consisted of 1:1 DMEM/F12 and Neurobasal media supplemented with 2 mM GlutaMax, 1XB27, 1XN2, 0.25% BSA, 0.1 mM non-essential amino acids, 25 U/ml penicillin/streptomycin (all products Invitrogen, Karlsruhe, Germany) and 20 ng/ml bFGF (Tebu-bio, Le Perray en Yvelines Cedex, France). Cells were propagated for 8–10 weeks with a medium change every 2–3 days, the formed clusters were dissected once per week. After this time period, the vast majority of cells expressed NSC markers like nestin and Sox 2. Cells were then frozen. For experiments, human NSC were thawn and single cells were seeded on culture surfaces that were coated with poly-L-ornithine (PLO; 0.001%; Sigma-Aldrich, Munich, Germany) and fibronectin (20 µg/ml; BioPur, Bubendorf, Switzerland). In parallel, we used the neuroectodermally pre-differentiated hNSC line (hNSC H9) for comparison. HNSC H9 cells were cultivated and propagated in the same way and in the same media as described above. Human NSCs derived from the cell line H9 were cultivated as free floating clusters and were dissected to single cells for experiments. The attached cells were either treated with human recombinant IFNγ (1000 U/ml; Millipore, Billerica, USA) or kept under control conditions for 3 days. This amount is equal to 100 ng/ml recombinant protein (murine and human). For differentiation experiments, cells had a day of recovery after seeding, then bFGF was withdrawn from the cells and differentiation was performed up to 3 months.

### IMMUNOCYTOCHEMISTRY

For immunocytochemistry, cells were seeded on coated cover slips (VWR International, Darmstadt, Germany). After 3 days under the influence bFGF (20 ng/ml both Tebu-bio) and under different treatments as indicated, the cells were fixed with 4% PFA (Roti-Histofix, Carl Roth, Karlsruhe, Germany) for 15 min at room temperature. Cells were blocked for 30 min at room temperature with 1 fold Roti-Immuno-Block containing 0.25% Triton X-100 for cell wall permeabilization (Carl Roth, Karlsruhe, Germany). Primary antibodies used at 4°C overnight were against βIII-tubulin (Tuj1; 1:500; R&D Systems, Minneapolis, USA), glial fibrillary acid protein (GFAP) (1:500; Dako, Hamburg, Germany), Sox2 (1:50; R&D Systems, Minneapolis, USA), IFNγ-R1 (1:500 Santa Cruz Biotechnology, Heidelberg, Germany), IFNγ-R2 (1:500; Santa Cruz Biotechnology, Heidelberg, Germany) and nestin (1:200; Covance, Munich, Germany). For detection of primary antibodies, fluoresceine-isothiocyanate (FITC; 1:500; Millipore, Billerica, USA) or indocarbocyanine (Cy3; 1:800; or Cy5; 1:200; Millipore, Billerica, USA) coupled secondary antibodies were used. The first and secondary antibodies were diluted in 1-fold Roti-Immuno-Block without Triton X-100 (Carl Roth, Karlsruhe, Germany). For visualization of cell nuclei cells were co-stained with DAPI (Invitrogen, Karlsruhe, Germany). For negative controls, primary antibodies were omitted in each experiment.

### MTT-ASSAY

To analyze the population size of hNSCs, the optical density, indicative of conversion of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) into formazan crystals which takes place in live cells only, was determined after IFNγ treatment at indicated concentrations. An OD value of 0.5 represents approximately 50,000 and an OD value of 1.0 represents approximately 100,000 live NSCs. The population size was measured after the indicated time intervals. Therefore, MTT (Invitrogen, Karlsruhe, Germany) was added to the cell culture media at a final concentration of 0.5 mg/ml after 5 h of incubation media was skipped and DMSO (Invitrogen, Karlsruhe, Germany)

was added for the solubilization of formed crystals. We seeded the same amount of cells at day 0 and performed an MTT-Assay on day 3, 5, and 7. At every time point control cells and cells treated with human recombinant IFNγ were tested. We used the same dose of human recombinant IFNγ, which was used in the murine study (1000 U/ml).

### REAL-TIME QUANTITATIVE PCR

RNeasy Kit (Qiagen, Hilden, Germany) was used for RNA isolation of cultured NSCs. Then a reverse transcription into cDNA (ABI, Darmstadt, Germany) was performed. Real-time quantitative PCR was carried out by the usage of the 7500 fast or 7500 real-time quantitative PCR cycler (ABI, Darmstadt, Germany). SYBR green master mix (Qiagen, Hilden, Germany) or equivalent chemistry from another supplier (Quantace, London, UK) was used. The specific primers for genes of interest or the housekeeping gene were either purchased (QuantiTect primer assays, Qiagen) or self-designed (BioTEZ, Berlin, Germany). The genes of interest (target gene) in IFNγ-treated groups or control groups (PBS-treated) were analyzed in at least 3 independent cultures in triplicate each. Every experiment in IFNγ-treated or control (PBS-treated) groups provided delta CT values (ΔCT: gene of interest minus reference gene).

### STATISTICAL ANALYSES

Experiments were repeated with independent cultures at least three times in triplicate each. The resulting data sets were statistically analyzed und illustrated using the GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA, 2003) software. For approval of statistical significance between groups, a two-tailed unpaired *t*-test was performed. *P*-values $<0.05$ were considered to indicate significant differences.

### CSF COLLECTION AND IFNγ ELISA

Lumbar CSF was obtained from patients admitted to our department for diagnostic purposes. Patients suffered from multiple sclerosis (three samples), peripheral nervous plexus affections (one samples), Alzheimer's disease (one sample), viral myelitis (one sample), or stroke (one sample), were compared to control CSF (three samples). All CSF specimens were immediately centrifuged, aliquoted and stored at –35°C. Ethics approval for the use of human CSF was obtained from the institutional ethics committee. For IFNγ detection in the CSF samples an IFNγ ELISA by R&D systems (R&D systems, Wiesbaden-Nordenstadt, Germany) was used. The experimental protocol followed the manufacturer's instructions. For statistical testing 3 control patients were compared with 3 MS patients with an unpaired two-tailed *t*-test.

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# 3. Result summary

## 3.1 A new role for interferon gamma in neural stem/precursor cell dysregulation

We were able to show, that the proinflammatory cytokine IFNγ leads to dysregulation in a substantial portion of murine NSPCs and murine NSCs. These findings were found in the undifferentiated stage of these cells, as well as in their differentiated progeny. The dysregulation is characterized by an unusual simultaneous expression of neuronal and glial markers (βIII-tubulin and GFAP) in undifferentiated cells. These cells show also a significant up-regulation of the mRNAs for this genes. Further, the gene-expression of SHH and Stat 1 (as a down-stream signaling molecule in the IFNγ pathway) was also significantly up-regulated. We were able to detect a decrease in the population extend and an up-regulation of caspases after IFNγ treatment. Also differentiated neural stem cells showed this dysregulated phenotype after IFNγ treatment. In parallel to the appearance of dysregulated cells, the amount of proper βIII-tubulin positive neurons decreased after IFNγ treatment. As, additionally, IFNγ-treated populations are not able to generate functionally active neuronal networks, the observed reduced neuronal differentiation might directly be linked to a functional deficit. Also on single cell level we were able to detect alterations, since msNSCs that were treated with IFNγ in their undifferentiated developmental phase showed aberrant electrophysiological properties after differentiation [69].

## 3.2 Interferon gamma and sonic hedgehog signaling are required to dysregulate murine neural stem/precursor cells

To further study the underlying mechanisms that lead to the dysregulated geno- and phenotype of msNSCs and msNSPCs after IFNγ exposure, we tried to antagonize sonic hedgehog (SHH) signaling with cyclopamine, since we found SHH mRNAs to be significantly up-regulated after IFNγ treatment. We speculated, that the up-regulation of SHH as major morphogene in brain development might be involved in

the alteration of cellular behavior. We were able to show, that the dysregulation of msNSPCs was significantly ameliorated by the pharmacological inhibition of this pathway and confirmed this on gene-expression and protein level. As a next step, we proved, if the up-regulation of SHH and Stat 1 (which was also significantly up-regulated after IFNγ treatment) was correlated to the amount of IFNγ. We could show, that the expression levels of both genes were correlated to the administered amount of IFNγ. After this findings we treated msNSPCs with murine recombinant SHH in high doses, to test if we could induce the dysregulated phenotype. We were not able to induce a dysregulation of msNSPCs with SHH administration. Thus we hypothesized, that only a crosstalk of Stat 1 signaling and SHH signaling is able to induce the altered geno- and phenotype in msNSPCs. We performed chromatin immunprecipitation studies to verify a possible crosstalk of both pathways. It was possible to prove, that phosphorylated Stat 1 molecules are binding to a gamma activated side in the SHH promoter, leading to the dysregulated phenotype of msNSPCs after IFNγ treatment [70].

## 3.3 Species-dependent differences of embryonic stem cell-derived neural stem cells after Interferon gamma treatment

After investigating the impact and the molecular mechanisms involved in the dysregulation of murine neural stem cells that were treated with IFNγ, we decided to explore possible impacts of this cytokine on human neural stem cells (hNSCs) that were derived from human embryonic stem cells (hESCs). We first assured that this hNSCs express the receptors against IFNγ on their cell surface. To this purpose we used immunocytochemistry and quantitative real-time PCR approaches. We found out, that the human cells indeed express both receptor chains. However, if compared to mNSCs, there was a trend towards less copies of mRNA in hNSCs for both receptor sub-units in comparison to the mNSCs used in the previous studies. We then treated these cells with the same amount of human recombinant IFNγ that was used in the murine studies and analyzed if dysregulated cells were also detectable in the human counterparts. We were not able to detect the same

dysregulated phenotype in murine cells. Furthermore, the human cells did not show any decrease in their population extend after IFNγ treatment. Therefore we analyzed if hNSCs are in general able to react to IFNγ stimulation and analyzed common down-stream signaling genes that were shown to be up-regulated after IFNγ exposure of murine cells. Indeed, we were able to show, that typical down-stream genes of IFNγ signaling were significantly regulated after IFNγ treatment in human populations. To speculate about the physiological importance of our findings, we measured the concentration of IFNγ in cerebrospinal fluid (CSF) specimens, taken after lumbar punction from patients suffering from multiple sclerosis, peripheral nervous plexus affections, Alzheimer dementia, viral myelitis or stroke. We found that patients suffering from multiple sclerosis during an acute relapse showed a significant increase of IFNγ (three control samples vs. three MS samples). Due to the limitation of patient material, we could only screen single patients with other diseases (one patient per indicated disease). But also here we found a clear trend to up-regulation of IFNγ in the CSF [71].

# 4. Discussion

In the last few years various studies were aimed at elucidating the role of the pro-inflammatory cytokine IFNγ during neurodegenerative or neuroinflammatory conditions and its impact on NSPCs and NSCs [70]. In those reports Janus-faced properties of IFNγ were described. On the one hand, pro neurogenic effects of IFNγ on neural stem cell differentiation were described [37,38,39,40,41,70]. On the other, a number of publications report a negative effect on neuronal differentiation and neurite outgrowth [42,43,44,45,46,47,48,70]. A dualistic role of IFNγ was also seen when proliferation and differentiation of astrocytes was investigated [70,72,73,74].

We were able to clarify some effects of this cytokine on murine and human neural stem cells in three publications.

The results from our first investigation shed new light on the effects of IFNγ on neural stem cells. Until now, IFNγ-related up-regulation of βIII-tubulin was interpreted as a beneficial enhancement of neurogenesis [41,69,72,75]. We disproved this view in our first study showing that IFNγ instead promotes an abnormal NSPC-derived cellular phenotype that does not relate to classical neurons or astrocytes and that appears to be dysregulated in terms of functional and molecular properties [69]. IFNγ treatment leads to the expression of both, class III βtubulin and GFAP in ~40% of NSPC which is abnormal and, even after differentiation, not linked to mature neuronal or astrocytic electrophysiological function [69]. Class III βtubulin isotype is usually considered specific for post-mitotic neurons, and such aberrant expression has so far only been noted in gliomas [76,77] or dysregulated tumorigenic neural stem cells [69,78,79]. Walton and colleagues even report some unusual coexpression of βIII-tubulin and GFAP in tumorigenic neural stem cells, a phenomenon similar to that detected here after IFNγ treatment of regular NSPCs [69].

The aspect of IFNγ-mediated NSPC dysregulation is further substantiated by an up-regulation of SHH which is paralleled by down-regulation of Gli1 which has been reported to be consistently up-regulated in the course of SHH signaling [69,80]. As expression patterns of neurogenic niche morphogenes like SHH or Gli1 are generally tightly regulated during CNS development, its disturbance points to misguided development or again tumorigenesis [46,80]. Thus, for the first time, we directly illustrated a possible link between IFNγ, NSPCs and cellular abnormalities similar to that observed in tumor cells strongly supporting the view that inflammation might be involved in tumor generation via neural stem cells [69]. Additionally, the IFNγ-related down-regulation of iNOS in NSPC cultures is untypical as it is known that IFNγ normally induces iNOS [69,81]. Our electrophysiological findings illustrate the importance of an additional functional control of morphological / immunocytochemical observations as the up-regulation of βIII-tubulin in differentiated NSPC-derived cells, which was interpreted as enhanced neurogenesis in different studies [72,75], was not paralleled by neuronal electrophysiological behavior [69]. Further, the increase in $GFAP^-$/$\beta$III-$tubulin^+$ neurons after IFNγ

treatment of proliferating cultures was not significant and after differentiation under the impact of IFNγ we even found significantly less GFAP$^-$/βIII-tubulin$^+$ neurons [69]. Interestingly, a similar observation was described previously [44,69]. We found that those βIII-tubulin expressing cells that significantly increased in numbers after IFNγ treatment of proliferating or differentiating cultures were also GFAP positive and exhibited electrophysiological properties that were neither typical for mature astrocytes nor for neurons [69]. We demonstrated this by carefully correlating electrophysiological data of patched cells with their immunocytochemical phenotype [69]. These molecular and functional IFNγ effects on NSPCs indicate a profoundly compromised cell function or, alternatively, a new IFNγ-induced NSPC-derived neural cell of unknown function [69]. Interestingly, ectopic expression of IFNγ during early stages of CNS development induces medulloblastomas via SHH overexpression [46] pointing towards a general dysregulating effect of IFNγ on NSPCs during development or disease [69]. To investigate functional neural development under controlled conditions, with and without IFNγ treatment, we electrophysiologically measured the development of functional neuronal networks starting from ES cell derived immature neural precursor cell cultures [69]. Usually, network activity progressively develops over time as a result of a complex interaction of a multitude of factors that converge to an integrated functional entity [69,82]. It depends on efficient synapse formation and function of an entire neuronal population [69]. If using immature neural precursor populations as developmental starting point, basic aspects of functional neural development can be measured [69]. In contrast, mature ES cell-derived functional neuronal networks can be used to detect acute functional consequences due to changes in extracellular composition [69]. These investigations then affect already active neuronal networks [69]. For instance, they showed to reversibly alter their network function under the influence of different cerebrospinal fluid specimens [69,83]. We chose a paradigm in which the influence of IFNγ selectively affected the initial proliferation period of cultures that were subsequently held under normal differentiating conditions [69]. IFNγ-treated cultures showed a significantly impaired development of neuronal network function, impressively pointing to an IFNγ-related, profoundly altered functional development of neural

precursor populations [69]. Thus, we speculate that abnormally high IFNγ production during development and CNS diseases might impair functional neuronal development in fetal neurogenesis or adult regeneration and propose to inhibit IFNγ effects on NSPCs as a means to effectively support their developmental and regenerative potential [69].

We then tried to elucidate the mechanisms beyond murine NSPC dysregulation. As SHH and Stat 1 were strongly up-regulated after IFNγ exposure, these proteins were suspected to be involved in murine NSPC dysregulation.

We speculated that SHH, as one of the major morphogenes in brain development, is involved in the formation of $GFAP^+$/ $\beta$III-$tubulin^+$ cells and that Stat 1 might be involved in SHH up-regulation [70]. To substantiate this hypothesis, we first investigated if Stat 1 can bind to the SHH promoter. For this, we performed a chromatin immunprecipitation assay and confirmed that a phosphorylated dimer of Stat 1 is capable to enter the nucleus in order to bind to a GAS sequence in the SHH promoter region and therefore leads to an up-regulation of SHH [70]. This shows that IFNγ-related Stat 1 up-regulation is directly linked to the induced SHH pathway [70].

To investigate if an activated SHH pathway is directly involved in generating the IFNγ-induced phenotype, we antagonized the SHH pathway by administration of cyclopamine [70]. Cyclopamine is known to inhibit the activity and down-stream-signaling of smoothened by a binding-induced conformational change in the smoothened protein [70,84]. We were able to show that the IFNγ-induced dysregulation of NSPCs is significantly ameliorated after antagonizing SHH signaling indicating that SHH signaling is necessary to induce the dysregulated phenotype [70]. This finding was confirmed by immunocytochemistry and real-time quantitative PCR [70]. Notably, we were not able to completely block IFNγ-induced NSPC dysregulation by cyclopamine [70]. This could probably be due to the low concentration of cyclopamine as it was used in our study to prevent toxicity [70]. After identification of SHH as mediator for NSPC dysregulation, we added recombinant murine SHH in high doses to undifferentiated NSPCs, to clarify if the dysregulated phenotype can be induced solely via SHH signaling downstream from

IFNγ [70]. However, SHH alone was not able to induce NSPC dysregulation [70]. This indicates that the IFNγ-induced dysregulated phenotype is depending on activation of the SHH pathway and simultaneously on other IFNγ-related effects [70]. Thus, the dysregulated phenotype could either be induced via a simultaneous SHH- and Stat 1-activation of the SHH promoter or, for instance, via simultaneous SHH-activation of the SHH promoter and activation of common [16,85,86] IFN-regulated genes (IRGs) or IFN-stimulated genes (ISGs) by the JAK/Stat cytokine pathway [70]. We summarized this in the schematic drawing [70]. The IFNγ-activated site (GAS) element in the SHH promoter region might therefore be involved in SHH up-regulation or in a direct co-activation of the dysregulated phenotype [70].

The binding of phosphorylated Stat 1 to the SHH promoter after IFNγ exposure was previously shown by Sun et al. [70,87]. Interestingly, Sun and colleagues did not find a dysregulated phenotype or a reduced proliferation, but they described a clear up-regulation of SHH after IFNγ administration and they also describe a binding of Stat 1 to the SHH-promoter region [70]. Notably, the cells used by Sun and colleagues were granular neural precursor cells from postnatal mice, in contrast to NSPCs or the murine ES cell-derived neural stem cells used in our study [70]. This suggests, that effects mediated by IFNγ exposure do not only seem to depend on the examined brain region, but also on the developmental stage and the cell type investigated. It is even more surprising that Todoric and colleagues described a cross-talk of IFNγ and SHH in human and murine pre-adipocytes as well [70,88]. This leads to the hypothesis, that crosstalk of IFNγ and SHH is an important genetically conserved factor in cellular response to inflammatory signals, since this pathway is not limited to the murine species or a special cell type [70]. Taken together these findings of our second publication provide new evidence for the importance of pro-inflammatory signals in cell fade- and differentiation-decisions since SHH is an important morphogene in brain development and the neural stem cells niche [70,89]. Therefore, we claim a complex and diverse role of IFNγ as mediator of dysregulation in NSPCs [70].

As our previous investigations were based on murine cells, we then decided to extend the experiments to the human level. To this purpose we used neural stem

cells, that were generated from human embryonic stem cells (detailed information is given in the material and methods part of publication number three).

We found that IFNγ has species-dependent effects on embryonic stem cell-derived neural populations [71]. While this pro-inflammatory key player induces a striking dysregulation in murine neural stem cells (msNSCs) with an unusual coexpression of neuronal and glial markers, it has no such effects on human neural stem cells (hNSCs) although both cell populations express appropriate IFNγ receptors and up-regulate most of the classical down-stream signals like i-NOS, IRF-9, c-Myc, MHC 1 and Stat 1 [71]. As we found tendentially more copies of mRNA for the IFNγ receptor in msNSCs, probably more IFNγ molecules can bind to the receptor leading to a stronger induction of IFNγ-related down-stream effects in these cells [71]. Another explanation for the divergent reactions after IFNγ exposure might be a qualitatively different induction of down-stream signals [71]. While SHH is significantly up-regulated after IFNγ treatment in msNSC populations, it remains unaltered in hNSC populations [71]. Interestingly, published results of our group show that the IFNγ-induced dysregulated murine phenotype depends on both, Stat 1 and SHH signaling [70,71]. Thus, the lack of an important down-stream signal in the human situation might explain the species-dependent differences [71]. As it is known that human gliomas and tumorigenic neural stem cells express SHH [90] and comprise cells that simultaneously express neuronal and glial markers [76,77,78,79], this morphogene was associated with brain tumor formation and/or growth [71]. Interestingly, in mice a link between IFNγ and SHH signaling was observed as an ectopic expression of IFNγ was shown to induce medulloblastoma formation via SHH overexpression [46,71]. According to our results, the relevance of an IFNγ-SHH crosstalk concerning the human situation is now uncertain [71].

The general relevance of IFNγ-related effects on human neural stem cells remains to be explored [71]. That IFNγ can influence the differentiation of human neural stem cells was demonstrated recently [71,75]. In that study, immortalized hippocampal or striatal human neural stem/progenitor cells from 12-week-old fetal brains showed increased neurogenesis and MHC-1 expression after IFNγ exposure [71].

In the present study we additionally demonstrated that patient CSF contains high amounts of IFNγ during relapses in multiple sclerosis and probably also in other nervous system diseases [71]. As ependymal cells lack tight junctions, the CSF compartment exchanges neuroactive substances with the interstitial fluid of the brain parenchyma including neurogenic zones [71,91]. This might point to a possible anatomical relationship between IFNγ within the CSF and neural stem cells within ventricular zones substantiating the relevance of IFNγ effects on neural stem cells in humans [71].

Our results show that data collected on a murine genetic background cannot automatically be translated to the human situation [71].

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# 6. Figure list

# 7. Publication list

1) Wellen J, **Walter J**, Jangouk P, Hartung HP, Dihne M (2009)
Title: Neural precursor cells as a novel target for interferon-beta.
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2) **Walter J**, Honsek SD, Illes S, Wellen JM, Hartung HP, et al. (2011)
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3) **Walter J**, Hartung HP, Dihne M (2012)
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5) Schwarz CS, Ferrea S, Quasthoff K, **Walter J**, Görg B, Häussinger D, Schnitzler A, Hartung HP, Dihne M (2012)
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Title: Flavin mononucleotide-based fluorescent proteins function in mammalian cells without oxygen requirement
Journal: PLoS One
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7) Endres S, **Walter J**, Dihne M, Jaeger KE, Drepper T (2012)
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8) Quasthoff K, **Walter J**, Ferrea S, Dihne M (2012)
working title: Effect of cryopreservation on network activity of primary rat cortical neurons
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# 8. Abbreviation list

| | |
|---|---|
| µg | microgram |
| µl | microliter |
| bFGF/FGF-2 | basic fibroblast growth factor |
| BrdU | 5-bromo-2-deoxyuridine |
| BSA | bovine serum albumin |
| $CaCl_2$ | calcium chloride |
| CHIP | chromatin immunprecipitation |
| c-Myc | myelocytomatosis virus oncogene cellular homolog |
| CNS | central nervous system |
| $CO_2$ | carbon dioxide |
| CP | cyclopamine |
| CSF | cerebrospinal fluid |
| CY3 | indocarbocyanine |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| EGF | epidermal growth factor |
| ELISA | Enzyme-linked immunosorbent assay |
| FITC | fluoresceine-isothiocyanate |
| GAPDH | glyceraldehyde- 3-phosphate dehydrogenase |
| GAS | interferon gamma - activated site |
| GFAP | glial fibrillary acidic protein |
| HEPES | 2-(4-(2-Hydroxyethyl)- 1-piperazinyl)-ethansulfonsäure |
| hESCs | human embryonic stem cells |
| hNSCs | human neural stem cells |
| ICAM-1 | intercellular adhesion molecule 1 |
| IFN$\alpha$ | Interferon alpha |
| IFN$\beta$ | Interferon beta |
| IFN$\gamma$ | Interferon gamma |
| IFN$\gamma$-R1 | Interferon gamma receptor 1 |
| IFN$\gamma$-R2 | Interferon gamma receptor 2 |

| | |
|---|---|
| iNOS | inducible nitric oxide synthase |
| iPSCs | induced pluripotent stem cells |
| IRF-9 | interferon regulatory factor 9 |
| IRGs | IFN-regulated genes |
| ISGs | IFN-stimulated genes |
| JAK 1 and 2 | janus kinase 1 and 2 |
| KCl | potassium chloride |
| kHz | kilohertz |
| K-$MeSO_3$ | potassium methanesulfonate |
| KSR | knock out serum replacement |
| Lif | leukemia inhibitory factor |
| MEA | multi electrode array |
| mESCs | murine embryonic stem cells |
| Mg-ATP | magnesium adenosine triphosphate |
| $MgCl_2$ | magnesium chloride |
| MHC 1 and 2 | major histocompatibility complex 1 and 2 |
| mM | millimolar |
| mNSC | summary for the terms “msNSPCs + msNSCs” used in the third publication for better readability |
| mRNA | messenger ribonucleic acid |
| msNSCs | murine neural stem cells |
| msNSPC-d | differentiated murine neural stem/precursor cells |
| msNSPC-p | proliferating murine neural stem/precursor cells |
| msNSPCs | murine neural stem/progenitor or neural stem/precursor cells |
| MTT | 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide |
| MΩ | megaohm |
| NaCl | sodium chloride |
| Na-GTP | natrium guanosine triphosphate |
| $NaH_2PO_4$ | sodium hydrogen phosphate |
| $NaHCO_3$ | sodium hydrogen carbonate |
| nm | nanometer |

| | |
|---|---|
| nSFEBs | neural precursor cell-enriched, serum-free, floating embryoid body-like aggregates |
| $O_2$ | oxygen |
| OD | optical density |
| PDL | poly-D-lysine |
| PFA | paraformalin |
| PLO | poly-L-ornithine |
| PTCH | patched |
| SEM | standard error of mean |
| SHH | sonic hedgehog |
| SMO | smoothened |
| Sox2 | SRY (sex determining region Y)-box 2 |
| Stat 1 | signal transducers and activators of transcription family 1 and 2 |
| U/ml | units per milliliter |
| βIII-tubulin | class III βtubulin |

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# 10. Declaration/Erklärung

Hiermit erkläre ich, dass die vorliegende Arbeit von mir verfasst wurde, ohne die Nutzung unerlaubter Hilfe.

Diese Dissertation wurde bei keiner weiteren Institution eingereicht. Es erfolgten keine erfolglosen Promotionsversuche an anderen Institutionen.

Die endgültig veröffentlichte Version dieser Dissertation unterscheidet sich insoweit von der primären Version, als dass zwei vorläufige Versionen der damals eingereichten Publikationen, die in diese kumulative Arbeit eingegangen sind, durch die final veröffentlichten Publikationen ersetzt wurden. Dies geschah, um den Urheberrechten der Verlage zu entsprechen, in denen die Publikationen erschienen sind, und ändert nichts am Inhalt der Dissertation. Die Seitenzahlen, die Zitate und das Inhaltsverzeichnis wurden dementsprechend angepasst. Dies wurde vom Erstgutachter der Arbeit genehmigt.

Düsseldorf, den 25.11.2012

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Janine Walter