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

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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 (IFN γ) is a main pro-inflammatory cytokine and up-regulated during several neurological diseases. IFN γ 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 IFN γ 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 IFN γ was finally demonstrated by applying the microelectrode array technology. IFN γ 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 IFN γ 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. IFN γ 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 IFN γ -activated microglial cells induce neurogenesis [6], IFN γ 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 IFN γ 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 IFN γ develop medulloblastomas [13], indicating that IFN γ may also be involved in malignant transformation of neural precursor cells.

In the present study, we demonstrated that IFN γ induces an abnormal immunocytochemical phenotype in NSPCs with simultaneous expression of neuronal and glial markers. Furthermore, IFN γ led to a dysregulated gene expression as well as dysfunctional electrophysiological properties. Additionally, we finally present evidence that IFN γ 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 IFN γ compromises neurogenesis. Thus, its role during inflammatory processes should be reassessed and IFN γ suppression during brain pathology possibly supports functional neurogenesis.

Results

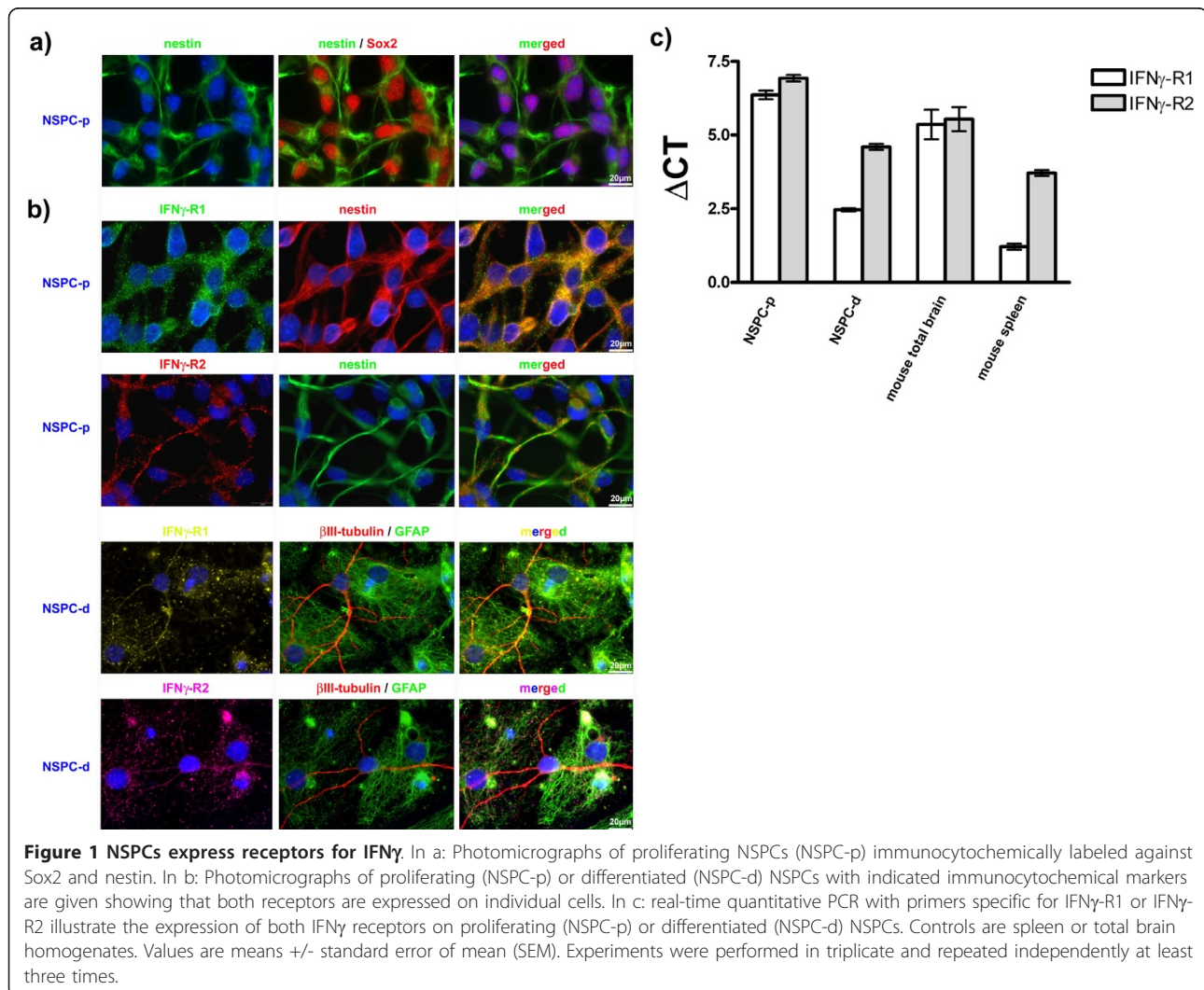
IFN γ receptors 1 and 2 are expressed in NSPCs and their differentiated progeny

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 β III-tubulin or GFAP (Figure 1B). The signal transduction process of the proinflammatory cytokine IFN γ starts with binding to the IFN γ 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 IFN γ 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.

IFN γ reduces the population extent of NSPCs

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



demonstrate that 100 or 1000 Units of IFN γ /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 IFN γ 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 IFN γ treatment. To detect possible anti-proliferative influences of IFN γ on NSPC populations, we performed BrdU labelings. Here, we were able to detect an additional slight but significant anti-proliferative effect of IFN γ (Figure 2D). Together, these data show that IFN γ exerts apoptotic and anti-proliferative effects on NSPCs that together lead to reduced population extents even under the influence of FGF-2.

IFN γ induces an abnormal phenotype in NSPCs

In the present study, we found that IFN γ treatment of proliferative murine E14 neurosphere-derived NSPCs caused up-regulation not only of transcripts for β III-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 predominantly pro-neurogenic effect of IFN γ . At the same time, IFN γ 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 IFN γ 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 IFN γ treatment (Figure 3B). Surprisingly, in addition to GFAP⁻/ β III-tubulin⁺ or GFAP⁺/ β III-tubulin⁻ cells, a considerable number of NSPCs ($39.3 \pm 14.5\%$ of all cells) co-expressed GFAP and β III-tubulin after a 3-day treatment with 1000 U/ml IFN γ (Figure 3B). GFAP⁺/ β III-tubulin⁺ cells in comparable numbers were also detectable after IFN γ treatment with only 100 U/ml (Figure 3B). Using a concentration of 100 U/ml IFN γ led to a slightly weaker induction of GFAP and β III-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 IFN γ (Figure 3B). In addition, IFN γ treatment induced simultaneous expression of GFAP and the post-mitotic neuronal markers MAP2a-c in a large percentage of cells (GFAP⁺/MAP2a-c⁺: $73.6 \pm 5.7\%$), illustrating that the co-

expression of glial and neuronal markers is not restricted to β III-tubulin. We next investigated the influence of IFN γ 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 β III-tubulin (Figure 4A). Moreover, numbers of GFAP⁺/ β III-tubulin⁺ or GFAP⁺/ β III-tubulin⁻ cells in IFN γ treated cultures differentiated for 7 days were significantly lower than without IFN γ (Figure 4A), which is in direct contrast to the expected pro-neurogenic role of IFN γ . 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 IFN γ . Also here we found GFAP⁺/ β III-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⁺/ β III-tubulin⁺ cells were induced even though the part of NS cell-derived GFAP⁺/ β III-tubulin⁺ cells was smaller in comparison to neurosphere-derived GFAP⁺/ β III-tubulin⁺ cells. This shows that IFN γ 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 IFN γ can drive the differentiation of NS cells or NSPC populations towards an immunocytochemically abnormal marker profile, indicating a genetic and/or functional dysregulation.

IFN γ induces an abnormal down-stream signaling in NSPCs

To study changes in IFN γ receptor expression, we performed quantitative real-time PCR after a 3-day IFN γ treatment of proliferative NSPCs. We detected increases in the transcript numbers of IFN γ -receptor1 and IFN γ -receptor2 (Figure 5A), as well as the IFN γ -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 IFN γ signaling, was down-regulated, revealing another surprising effect of IFN γ on NSPCs (Figure 5A). We investigated IFN γ -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 IFN γ does not promote neuronal determination, we found that Math1, Mash1, Neurogenin1 and Pax6 were down-regulated in NSPCs after IFN γ treatment (Figure 5B). Further, while IFN γ

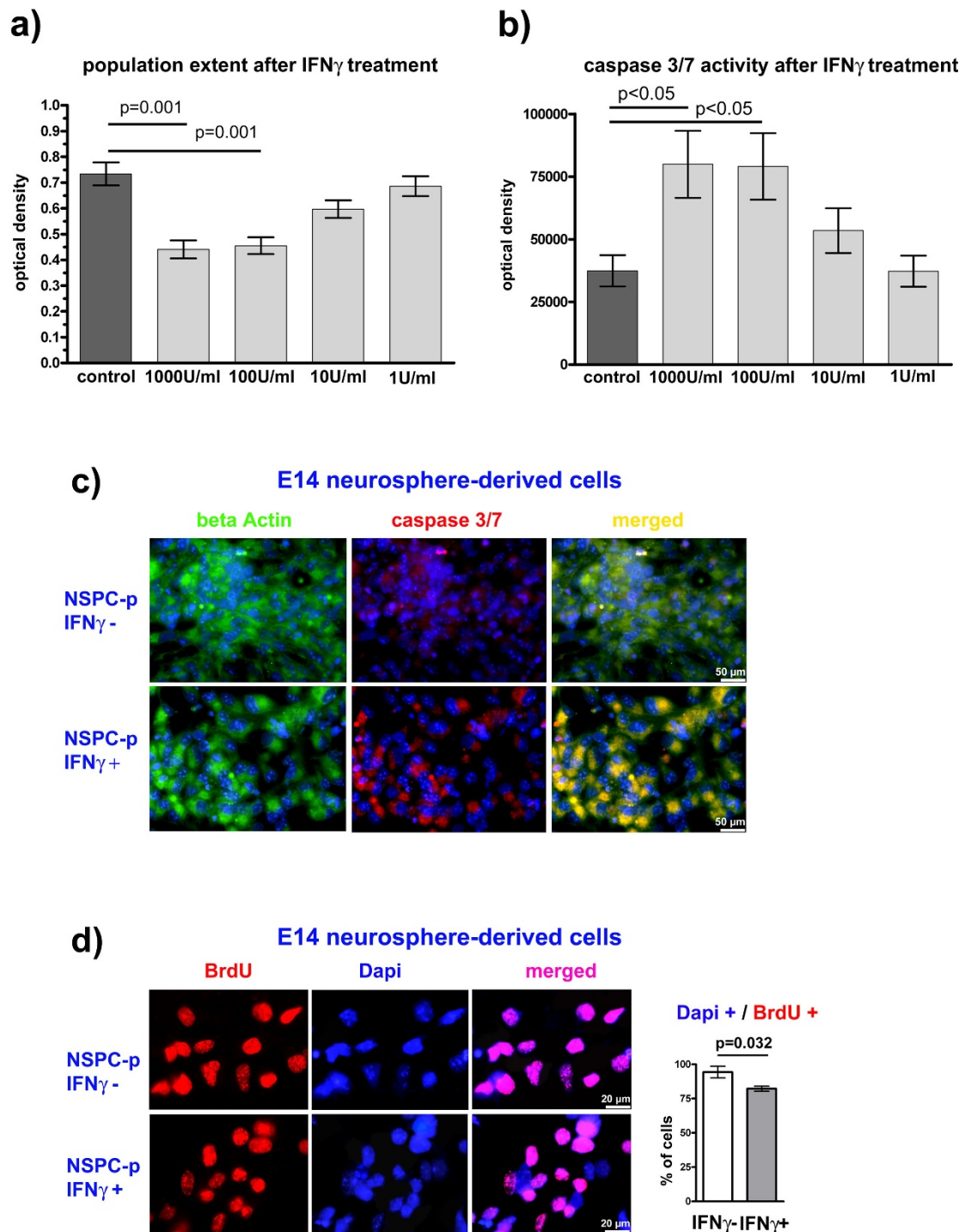


Figure 2 IFN γ reduces the population extent of NSPCs. In a: Optical densities [9] correlate to the extent of NSPC populations after a 48-hour IFN γ treatment with indicated concentrations. Values are means \pm standard error of mean (SEM). Experiments were performed in triplicate and repeated independently at least three times. In b: Optical densities correlate to caspase 3/7 activity after a 48-hour IFN γ treatment with indicated concentrations. Values are means \pm 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 IFN γ 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 IFN γ treatment. Additionally, quantification of BrdU $^+$ cells is given. Values are means \pm standard error of mean (SEM). Experiments were performed in triplicate and repeated independently at least three times. DAPI $^+$ nuclei are given in blue.

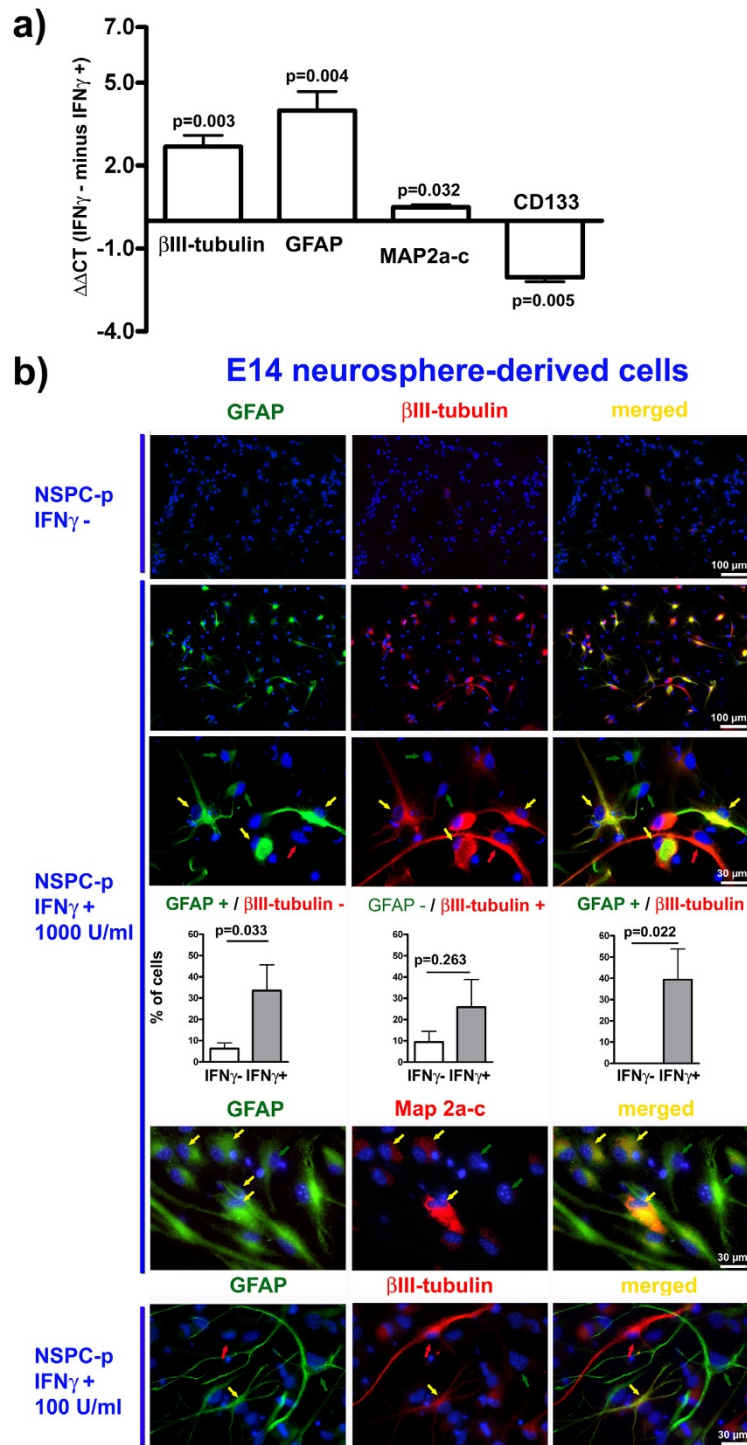


Figure 3 Cell type-specific marker expression after IFN γ treatment under proliferative conditions. In a: Results from real-time quantitative PCRs expressed as $\Delta\Delta$ CT (IFN γ - minus IFN γ +) of PBS-treated control and IFN γ -treated groups for the indicated markers of proliferating NSPCs.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

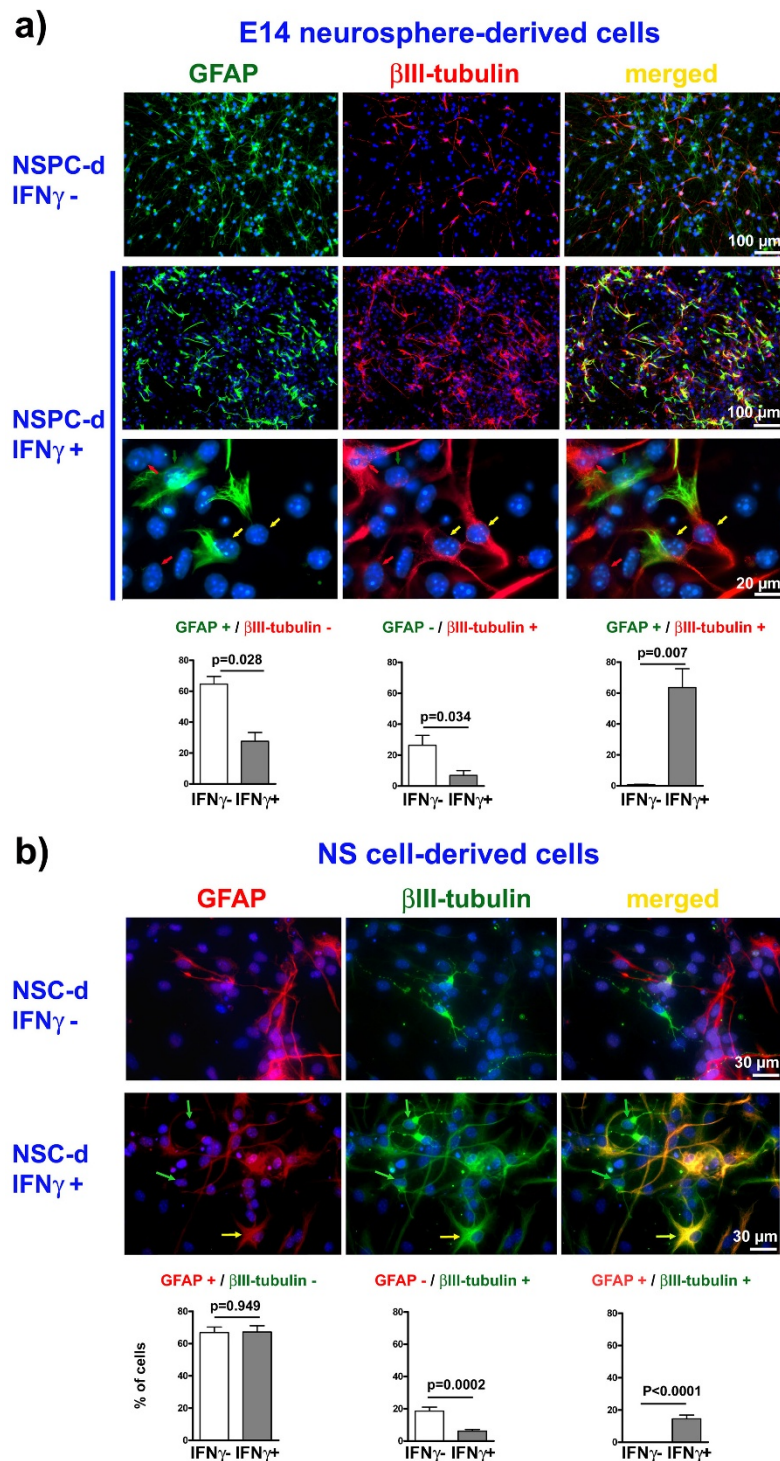
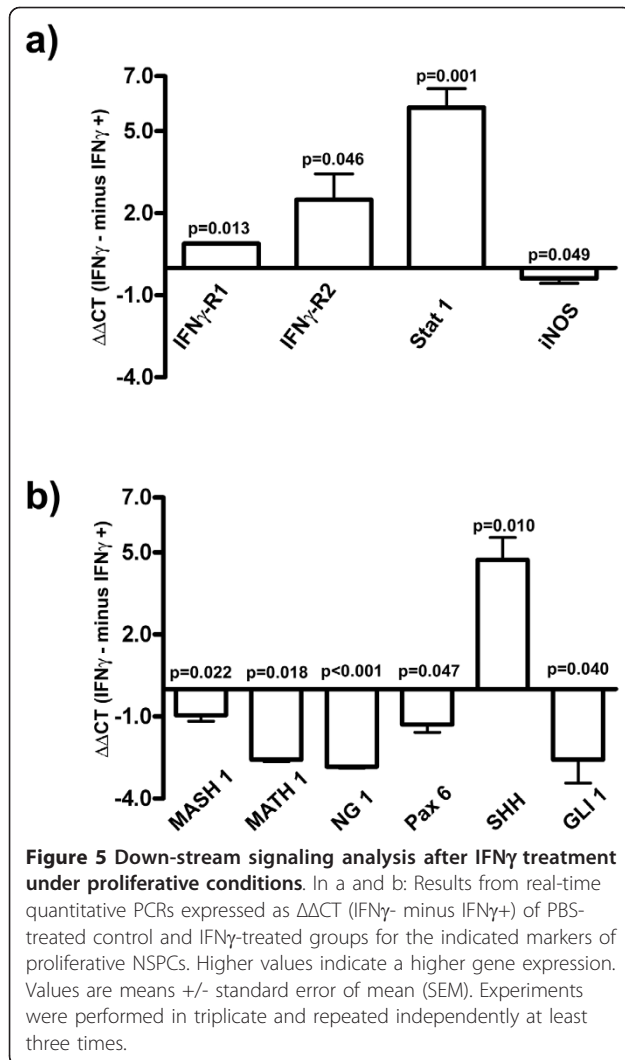


Figure 4 Cell type-specific marker expression after IFN γ treatment during differentiation. In a: Photomicrographs of NSPC populations after a differentiation period of 7 days (NSPC-d) are given with indicated immunocytochemical markers and the corresponding quantification of immuno-positive cells from all DAPI⁺ cells with (IFN γ +) or without (IFN γ -) IFN γ treatment as indicated. Values are means \pm 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 (IFN γ +) or without (IFN γ -) IFN γ treatment are given. Yellow arrows mark GFAP⁺/ β III-tubulin⁺ cells. Red arrows mark GFAP⁺/ β III-tubulin⁺ neurons and green arrows mark GFAP⁺/ β III-tubulin⁺ astrocytes. Note the untypical morphology of GFAP⁺/ β III-tubulin⁺ cells under IFN γ treatment in comparison to control. Values are means \pm 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 $^+$ / β III-tubulin $^+$ cells exhibit non-neuronal and non-astrocytic functional properties

To elucidate if the unusual effects of IFN γ treatment on the differentiation of NSPCs are accompanied by an atypical functional phenotype, we next analyzed basic electrophysiological properties of GFAP $^+$ / β III-tubulin $^+$ cells. For this purpose we combined whole-cell patch-clamp with subsequent immunocytochemistry to unambiguously identify recorded cells. GFAP $^+$ / β III-tubulin $^+$ cells in IFN γ -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 $^+$ / β III-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 $^+$ / β III-tubulin $^-$ cells in differentiated cultures either exhibited an outward rectifying IV relationship, similar to GFAP $^+$ / β III-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 $^+$ / β III-tubulin $^+$ cells. Our electrophysiological results, thus, demonstrate that GFAP $^+$ / β III-tubulin $^+$ cells are functionally distinct from mature astrocytes as well as neurons.

IFN γ treatment impaired the formation of in vitro functional neural networks

To ultimately verify the effect of IFN γ 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 IFN γ during their initial 7-day proliferation period under the influence of FGF-2. Hereafter, IFN γ was removed and differentiation was initiated by FGF-2 withdrawal. By this paradigm, IFN γ treatment selectively hit developmental processes of ES cell-derived NSPCs, while subsequent synapse formation and other maturational processes were excluded from direct IFN γ influences. After the initial 7-day period, maturation of cultures was observed for additional 44 days. Normally, during the 3rd and 4th 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, IFN γ -). 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 IFN γ 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 IFN γ -treated conditions. However, even though IFN γ 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 IFN γ on NSPCs. Until now, IFN γ -related up-regulation of

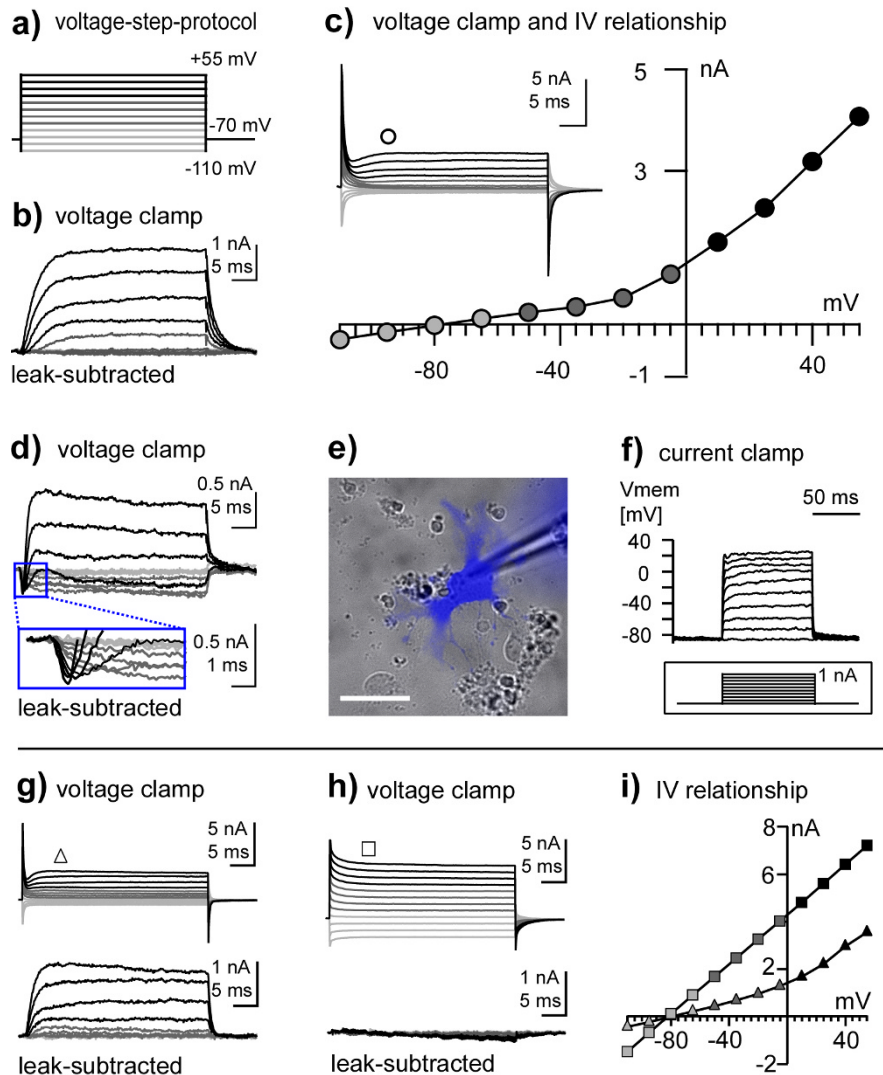
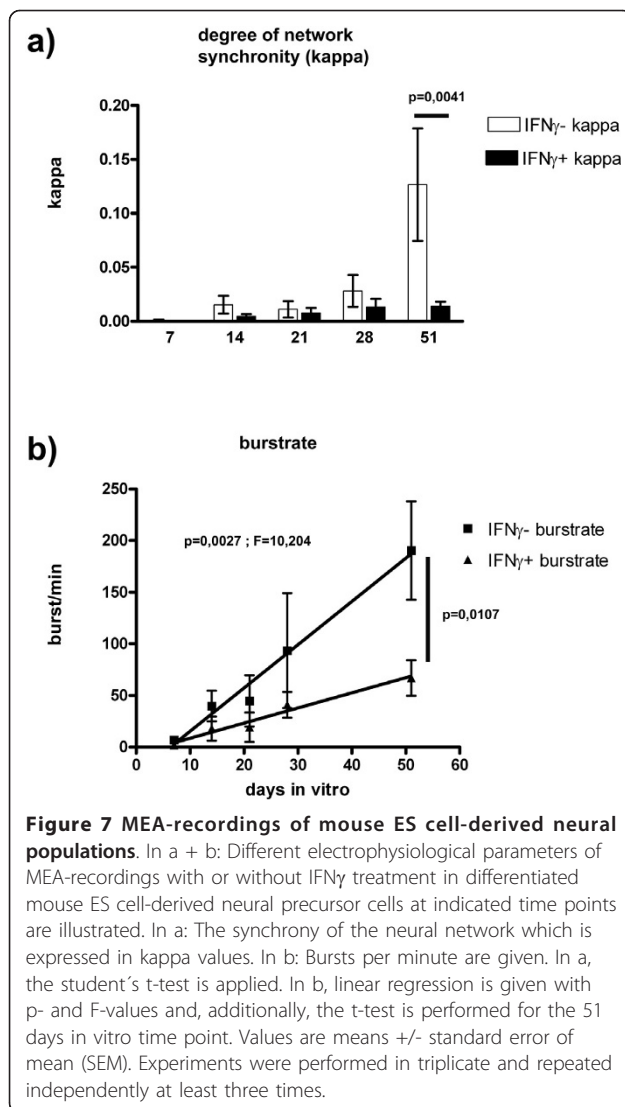


Figure 6 Patch-clamp results of IFN γ treated NSPCs. In a: Voltage-step protocol used to probe cells for voltage-activated conductances (see current traces in panels b-d, g, and h). In b, c: GFAP $^+$ / β III-tubulin $^+$ cells typically exhibited an outward rectifying current-voltage (IV) relationship (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 $^+$ / β III-tubulin $^+$ cells, voltage-gated inward currents were observed in addition to outward currents. In e: Image of cell filled with Alexa Fluor 350 and later identified as GFAP $^+$ / β III-tubulin $^+$ (scale bar 30 μ m, recordings illustrated in figure d) and f). In f: None of the 14 GFAP $^+$ / β III-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 $^+$ / β III-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).

β III-tubulin was interpreted as a beneficial enhancement of neurogenesis [7-9]. The present study disproves this view and shows that IFN γ 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. IFN γ treatment leads to the expression of both, class III β tubulin and GFAP in \sim 40% of NSPC which is abnormal and, even after differentiation, not linked to mature neuronal or astrocytic electrophysiological function. Class III β tubulin 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 β III-tubulin and GFAP in tumorigenic neural stem cells, a phenomenon similar to that detected here after IFN γ treatment of regular NSPCs. The aspect of IFN γ -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



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 IFN γ , 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 IFN γ -related down-regulation of iNOS in NSPC cultures is untypical as it is known that IFN γ normally induces iNOS [20]. Our electrophysiological findings illustrate the importance of an additional functional control of morphological/immunocytochemical observations as the up-regulation of β III-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 $^+$ / β III-tubulin $^+$ neurons after IFN γ treatment of proliferating cultures was not significant in the present study and after differentiation under the impact of IFN γ we even found significantly less GFAP $^+$ / β III-tubulin $^+$ neurons. Interestingly, a similar observation was described previously [21]. We found that those β III-tubulin expressing cells that significantly increased in numbers after IFN γ 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 IFN γ effects on NSPCs indicate a profoundly compromised cell function or, alternatively, a new IFN γ -induced NSPC-derived neural cell of unknown function. Interestingly, ectopic expression of IFN γ during early stages of CNS development induces medulloblastomas via SHH overexpression [13] pointing towards a general dysregulating effect of IFN γ on NSPCs during development or disease.

To investigate functional neural development under controlled conditions, with and without IFN γ -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 IFN γ selectively affected the initial proliferation period of cultures that were subsequently held under normal differentiating conditions. IFN γ -treated cultures showed a significantly impaired development of neuronal network function, impressively pointing to an IFN γ -related, profoundly altered functional development of neural precursor populations.

Conclusion

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

functional neuronal development in fetal neurogenesis or adult regeneration and propose to inhibit IFN γ 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 μ l 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].

IFN γ 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 μ g/ml; Tebu-bio) coated cover slips (VWR International, Darmstadt, Germany) at a density of 50×10^3 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 IFN γ treated conditions, cultures were kept under the influence of EGF/FGF-2 without or with IFN γ (1000 U/ml; Millipore) until fixation after further 3 days (NSPC-p -IFN γ /+IFN γ). 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 IFN γ until fixation (NSPC-d -IFN γ /+IFN γ). 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), β III-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), IFN γ -R1 (1:500; Santa Cruz Biotechnology) and beta Actin (1:100; Millipore) and polyclonal rabbit antibodies to glial fibrillar acid protein (GFAP) (1:500; Dako, Hamburg, Germany or 1:1000; Abcam), caspase (1:100; Cell Signaling), IFN γ -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 IFN γ 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 IFN γ treatment as indicated.

Caspase-activity Assay

For detection of caspase 3/7 activity after IFN γ treatment, we used the Caspase-Glo 3/7 assay (Promega, Madison, USA). Proliferating cultures were treated with decreasing concentrations of IFN γ 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 IFN γ -treated groups or control groups (PBS-treated) were analyzed in at least 3 independent cultures in triplicate each. Every experiment in IFN γ -treated or control (PBS-treated) groups provided delta CT values (Δ CT: gene of interest minus housekeeping gene). The presented graphs are $\Delta\Delta$ CT values:

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

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 M Ω when filled with intracellular solution containing (in mM): 120 K-MeSO₃, 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 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 20 glucose, bubbled with 95% O₂ and 5% CO₂ 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 β tubulin immunocytochemistry. By means of fluorescence at 350 nm electrophysiologically recorded cells were identified and assigned either to GFAP⁺/ β III-tubulin⁺ or GFAP⁺/ β III-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 μ g/ml, Sigma-Aldrich, Germany) and laminin (15 μ g/ml, Sigma-Aldrich, Germany) coated MEAs with a square grid of 60 planar Ti/TiN electrodes (30- μ m diameter, 200- μ m spacing) and an input impedance of <50 k Ω 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 and 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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References

1. Bjorklund A, Lindvall O: Cell replacement therapies for central nervous system disorders. *Nat Neurosci* 2000, **3**:537-544.
2. Lucas SM, Rothwell NJ, Gibson RM: The role of inflammation in CNS injury and disease. *Br J Pharmacol* 2006, **147**(Suppl 1):S232-240.
3. Schmitz T, Chew LJ: Cytokines and myelination in the central nervous system. *ScientificWorldJournal* 2008, **8**:1119-1147.
4. Liesz A, Suri-Payer E, Veltkamp C, Doerr H, Sommer C, Rivest S, Giese T, Veltkamp R: Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nat Med* 2009, **15**:192-199.
5. Lees JR, Golumbek PT, Sim J, Dorsey D, Russell JH: Regional CNS responses to IFN-gamma determine lesion localization patterns during EAE pathogenesis. *J Exp Med* 2008, **205**:2633-2642.
6. Butovsky O, Ziv Y, Schwartz A, Landa G, Talpalar AE, Pluchino S, Martino G, Schwartz M: Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. *Mol Cell Neurosci* 2006, **31**:149-160.
7. Whitney NP, Eidem TM, Peng H, Huang Y, Zheng JC: Inflammation mediates varying effects in neurogenesis: relevance to the pathogenesis of brain injury and neurodegenerative disorders. *J Neurochem* 2009, **108**:1343-1359.
8. Baron R, Nemirovsky A, Harpaz I, Cohen H, Owens T, Monsonego A: IFN-gamma enhances neurogenesis in wild-type mice and in a mouse model of Alzheimer's disease. *Faseb J* 2008, **22**:2843-2852.
9. Johansson S, Price J, Mado M: Effect of inflammatory cytokines on major histocompatibility complex expression and differentiation of human neural stem/progenitor cells. *Stem Cells* 2008, **26**:2444-2454.
10. Wang J, Lin W, Popko B, Campbell IL: Inducible production of interferon-gamma in the developing brain causes cerebellar dysplasia with activation of the Sonic hedgehog pathway. *Mol Cell Neurosci* 2004, **27**:489-496.
11. LaFerla FM, Sugarman MC, Lane TE, Leisring MA: Regional hypomyelination and dysplasia in transgenic mice with astrocyte-directed expression of interferon-gamma. *J Mol Neurosci* 2000, **15**:45-59.
12. Kim IJ, Beck HN, Lein PJ, Higgins D: Interferon gamma induces retrograde dendritic retraction and inhibits synapse formation. *J Neurosci* 2002, **22**:4530-4539.
13. Lin W, Kemper A, McCarthy KD, Pytel P, Wang JP, Campbell IL, Utset MF, Popko B: Interferon-gamma induced medulloblastoma in the developing cerebellum. *J Neurosci* 2004, **24**:10074-10083.
14. Zhou M, Schools GP, Kimelberg HK: Development of GLAST(+) astrocytes and NG2(+) glia in rat hippocampus CA1: mature astrocytes are electrophysiologically passive. *J Neurophysiol* 2006, **95**:134-143.
15. Ignatova TN, Kukekov VG, Laywell ED, Suslov ON, Vrionis FD, Steindler DA: Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *Glia* 2002, **39**:193-206.
16. Katsetos CD, Del Valle L, Geddes JF, Assimakopoulou M, Legido A, Boyd JC, Balin B, Parikh NA, Maraziotis T, de Chadarevian JP, et al: Aberrant localization of the neuronal class III beta-tubulin in astrocytomas. *Arch Pathol Lab Med* 2001, **125**:613-624.
17. Walton NM, Snyder GE, Park D, Kobeissy F, Scheffler B, Steindler DA: Gliotypic neural stem cells transiently adopt tumorigenic properties during normal differentiation. *Stem Cells* 2009, **27**:280-289.
18. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB: Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003, **63**:5821-5828.
19. Stecca B, Ruiz i Altaba A: Brain as a paradigm of organ growth: Hedgehog-Gli signaling in neural stem cells and brain tumors. *J Neurobiol* 2005, **64**:476-490.
20. Bach EA, Aguet M, Schreiber RD: The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu Rev Immunol* 1997, **15**:563-591.
21. Wang Y, Imitola J, Rasmussen S, O'Connor KC, Khoury SJ: Paradoxical dysregulation of the neural stem cell pathway sonic hedgehog-Gli1 in autoimmune encephalomyelitis and multiple sclerosis. *Ann Neurol* 2008, **64**:417-427.
22. Illes S, Theiss S, Hartung HP, Siebler M, Dihne M: Niche-dependent development of functional neuronal networks from embryonic stem cell-derived neural populations. *BMC Neurosci* 2009, **10**:93.
23. Otto F, Illes S, Opatz J, Laryea M, Theiss S, Hartung HP, Schnitzler A, Siebler M, Dihne M: Cerebrospinal fluid of brain trauma patients inhibits in vitro neuronal network function via NMDA receptors. *Ann Neurol* 2009, **66**:546-555.
24. Okabe S, Forsberg-Nilsson K, Spiro AC, Segal M, McKay RD: Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. *Mech Dev* 1996, **59**:89-102.
25. Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, Biella G, Sun Y, Sanzone S, Ying QL, Cattaneo E, Smith A: Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol* 2005, **3**:e283.

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