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Glucocorticoids attenuate interleukin-6-induced c-Fos and Egr1 expression and impair neuritogenesis in PC12 cells

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Abstract

Interleukin-6 (IL-6) is a cytokine primarily known for immune regulation. There is also growing evidence that IL-6 triggers neurogenesis and impacts neural development, both life-long occurring processes that can be impaired by early-life and adult stress. Stress induces the release of glucocorticoids by activation of the hypothalamic–pituitary–adrenal (HPA) axis. On the cellular level, glucocorticoids act via the ubiquitously expressed glucocorticoid receptor. Thus, we aimed to elucidate whether glucocorticoids affect IL-6-induced neural development. Here, we show that IL-6 signalling induces neurite outgrowth in adrenal pheochromocytoma PC12 cells in a mitogen-activated protein kinase (MAPK) pathway-dependent manner, since neurite outgrowth was diminished upon Mek-inhibitor treatment. Using quantitative biochemical approaches, such as qRT-PCR analysis of Hyper-IL-6 treated PC12 cells, we show that neurite outgrowth induced by IL-6 signalling is accompanied by early and transient MAPK-dependent mRNA expression of immediate early genes coding for proteins such as early growth response protein 1 (Egr1) and c-Fos. This correlates with reduced proliferation and prolonged G0/G1 cell cycle arrest as determined by monitoring the cellular DNA content using flow cytometry. These results indicate for IL-6 signalling-induced neural differentiation. Interestingly, the glucocorticoid Dexamethasone impairs early IL-6 signalling-induced mRNA expression of c-Fos and Egr1 and restrains neurite outgrowth. Impaired Egr1 and c-Fos expression in neural development is implicated in the aetiology of neuropathologies. Thus, it appears likely that stress-induced release of glucocorticoids, as well as therapeutically administered glucocorticoids, contribute to the development of neuropathologies by reducing the expression of Egr1 and c-Fos, and by restraining IL-6-dependent neural differentiation.

Abbreviations: AEBSF, 4-(2-Aminoethyl)-benzolsulfonylfluorid; Arc, activity-regulated cytoskeleton-associated protein; BDNF, brain-derived neurotrophic factor; Dex, dexamethasone; Egr1, early growth response protein 1; Erk, extracellular signal regulated kinase; FBS, fetal bovine serum; Gab1, Grb2-associated binder 1; GC, glucocorticoid; GR, glucocorticoid receptor; Grb2, growth factor receptor bound protein 2; GRE, glucocorticoid responsive element; HPA, hypothalamic–pituitary–adrenal; HPRT1, hypoxanthine-guanine phosphoribosyltransferase 1; Hy-IL-6, Hyper-interleukin-6; IEG, immediate early gene; IL-6, interleukin-6; Jak, Janus kinase; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; PBS, phosphate-buffered saline; PH, pleckstrin homology; PI3K, phosphatidyl-inositol-3-kinase; PIP3, phosphatidylinositol (3:4:5)-trisphosphate; PKB/Akt, protein kinase B; RPMI, Roswell Park Memorial Institute; RRID, Research Resource Identifier (see scicrunch.org); SHP2, Src-homology domain containing phosphatase; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription.

KEYWORDS

dexamethasone, glucocorticoids, interleukin-6, neural differentiation, neuropathology, stress

1 | INTRODUCTION

Interleukin-6 (IL-6) is a pleiotropic cytokine acting on immune regulation and is particularly involved in inflammatory processes (for review, see (Heinrich et al., 1998)). Hence, IL-6 secretion and IL-6 signalling are tightly controlled to maintain its physiological role in immune regulation. IL-6 signals via its receptor complex comprised of the IL-6-receptor (IL-6R) and gp130. Receptor activation initiates intracellular signalling pathways such as the Janus kinase (Jak)/signal transducer and activator of transcription (STAT) cascade and the extracellular signal regulated kinase (Erk)/mitogen-activated protein kinase (MAPK) cascade. As IL-6 signalling coordinates the immune response, dysregulated IL-6 signalling is associated with autoimmune diseases, such as rheumatoid arthritis or multiple sclerosis (for review, see (Heinrich et al., 2003)).

Besides its physiological and pathophysiological effects on immune regulation, IL-6 impacts neural development via Jak/STAT and MAPK/Erk pathway activation. IL-6 contributes to adult neurogenesis and induces differentiation of neural progenitor cells derived from induced pluripotent stem cells (Sulistio et al., 2018) (for reviews, see Heese, 2017; Islam et al., 2009)). Mice lacking IL-6 show decreased neural progenitor maturation in neurogenic brain regions such as the hippocampal dentate gyrus compared to their wild-type littermates (Bowen et al., 2011). Additionally, IL-6 signalling induces neurite outgrowth of rat pheochromocytoma PC12 cells, a well-characterized model for neural differentiation (Ihara et al., 1997; März et al., 1997; Satoh et al., 1988; Valerio et al., 2002; Wu & Bradshaw, 1996). However, IL-6 is also associated with a variety of neuropathologies (for review, see (Erta et al., 2012)). All these activities highlight the impact of IL-6 on neural differentiation and brain development.

Neural differentiation and brain development crucially depend on the expression of mRNA of immediate early genes (IEGs) coding for proteins such as c-Fos, early growth response protein 1 (Egr1), and activity-regulated cytoskeleton-associated (Arc) protein also known as Arg3.1 (for reviews, see Duclot & Kabbaj, 2017; Gallo et al., 2018)). The expression of these IEGs is induced by neural activity or cytokines such as brain-derived neurotrophic factor (BDNF) or nerve growth factor (NGF) (Alder et al., 2003; Wibrand et al., 2006). Furthermore, IEG mRNA expression and thus neural development and activity are affected by environmental factors such as stress (Jafari et al., 2017) (for reviews, see McEwen, Bowles, et al., 2015; McEwen et al., 2015; Reul, 2014)). Stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis leads to glucocorticoid release, resulting in glucocorticoid receptor (GR) activation in various cell types such as neurons (for reviews, see Reul & de Kloet, 1985; Wang et al., 2013)). Glucocorticoids and the activation of the GR in neurons affect neural differentiation and survival, thus participating in brain development and function (Glick et al., 2000; Oitzl et al., 1997; Pugh et al., 1997) (for review, see Gould & Tanapat, 1999; Liu et al., 1997; Odaka et al., 2017)). Hence, a high glucocorticoid level induced by stress in pre- or postnatal early-life provides a crucial programming factor for the development of neuronal circuits and is associated with neuropsychiatric disorders such as depression, anxiety, or schizophrenia (Bock et al., 2014; Novaes et al., 2017) (for reviews, see Majcher-Maslanka et al., 2018; Meyer & Feldon, 2010)). Additionally, glucocorticoids regulate adult neurogenesis in the dentate gyrus (Cameron & Gould, 1994; Gould et al., 1992). Neurogenesis in adulthood is attenuated by chronic stress and GR hyper-activation, contributing to the development of mood disorders (Duman et al., 2001; Pham et al., 2003; Sousa et al., 1999). Hence, perinatal as well as adult exposure to stress enhances glucocorticoid levels, induces GR activation, and negatively affects neural development.

Synthetic glucocorticoids such as Dexamethasone (Dex), are widely used for a long time to suppress immune activation and reduce inflammation, for example, in course of a pharmacological treatment of rheumatoid arthritis or multiple sclerosis (Cohen et al., 1960; McFarland, 1969). Dex is also used to prevent pregnancy complications, such as pre-term birth and reduces neonatal respiratory distress syndrome by promoting fetal lung maturation (Crowther et al. 2015; Health 1994) (for review, see (Haram et al., 2017)). However, there is evidence that exposure to Dex impairs neural differentiation (Moors et al., 2012) and brain functions such as learning and memory, and is associated with neuropsychiatric disorders, like depression or schizophrenia (Davis et al., 2013; Thompson et al., 2001) (for reviews, see Celano et al., 2011; Hunter et al., 2016)). Thus, GR hyper-activation either by stress or by synthetic glucocorticoids impairs brain development and functions.

In this study, we shed light on the molecular impact of the synthetic glucocorticoid Dex on IL-6-induced signalling and how IL-6-induced neural differentiation is impaired by Dex. Our results will contribute to understanding impaired neural development and brain functions associated with various neuropsychiatric disorders in response to Dex treatment or stress.

2 | MATERIAL AND METHODS

2.1 | Materials

The activation/phosphorylation-specific antibodies for tyrosine 627-phosphorylated Gab1 (RRID:AB_2107683, #3233), threonine 202- and tyrosine 204-phosphorylated Erk1/2 (RRID:AB_2315112, #4370), tyrosine 705-phosphorylated STAT3 (RRID:AB_331586, #9131), serine 473-phosphorylated Akt (RRID:AB_2315049, #4060), Akt (RRID:AB_2225340, #4685), and the antibodies specific for Erk1/2 (RRID:AB_390780, #4696), STAT3 (RRID:AB_331757, #9139), and GR (RRID:AB_2631286, #12041) were from Cell

Signaling Technology (Germany). Gab1 recognizing antibody (RRID:AB_310182, #06–579) was purchased from Millipore (USA). Antibody against SOCS3 (RRID:AB_494500, #18391) was from Immuno-Biological Laboratories (Japan). MEK inhibitor U0126 (#9903, 2019, Cell Signaling Technology) was dissolved in DMSO (A994.2, 2013, Roth, Germany). Dex (#D4902, 2010, Merck) and RU-486 (#M8046, 2010, Merck) were dissolved in 100% ethanol. RPMI-1640 (phenol red-free) medium (#11835-063, 2020), penicillin/streptomycin (#15140-122, 2020), and Fetal bovine serum (FBS, #10270-106, 2015) were from Gibco Life Technologies (Germany). Hyper-IL-6 (Hy-IL-6) was a generous gift from Stefan Rose-John (Christian-Albrechts-University, Kiel) (Fischer et al., 1997).

2.2 | Cell culture

Fully authenticated PC12 cells (RRID:CVCL_0481, ACC 159, DSMZ, Germany) were freshly purchased for this project in 11/2019. This cell line is not listed as commonly misidentified cell line by the International Cell Line Authentication Committee (https://protect-au.mimecast.com/s/kRfFCMwvygsqVkWR8SwirAw?domain=iclac.org/). Cells were maintained confluently in phenol-red free RPMI-1640 supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/L), and penicillin (60 mg/L) in a water-saturated atmosphere in 37°C and 5% CO_2. Maximal number of passages was 20. For experiments, confluent PC12 cells were seeded on poly-L-lysin (#P4707, 2019, Merck, Germany)-coated tissue culture dishes to allow adherence.

2.3 | Neurite outgrowth quantification

For quantification of neurite outgrowth of PC12 cells, pictures were taken from three arbitrarily selected fields per dish at the times, indicated. Phase-contrast images were obtained using the EVOS FL imaging system (ThermoFisher Scientific) and an objective providing 20x magnification. Neurite length was determined with ImageJ (https://imagej.nih.gov/ij/).

2.4 | Western blotting

Cells were lysed in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40/Igepal, 15% glycerol), supplemented with NaF (1 mM), Na_3VO_4 (1 mM), AEBSF (0.8 µM) (#2931.2, 2019, Roth) and aprotinin (#A162.1, 2019, Roth), pepstatin (#2936.2, 2016, SigmaAldrich), leupeptin (#CN33.3, 2016, MP Biochemicals) (10 µg/ml of each). Protein concentrations of lysates were determined by Biorad Protein Assay (#500-0006, 2015, Biorad). Equal quantities of protein were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. After blocking with Roti®Block (#A151.4, 2017, Roth, Germany), membranes were incubated with specific primary antibodies (1:1,000) and subsequently incubated with a secondary IRDye 800CW-conjugated anti-rabbit (# 925–32211, 2019, LI-COR, USA) or IRDye 680RD-conjugated anti-mouse antibody (#925-68070, 2019, LI-COR, USA). Proteins were visualized using the LI-COR Odyssey Infrared Imaging System (LI-COR, USA). Quantification of signal intensities was performed with Image Studio™ Lite software (LI-COR Bioscience, USA). For quantification of phosphorylation, signal intensities of phosphorylated species were normalized to signals from the corresponding total species.

2.5 | qRT-PCR

Total RNA of PC12 cells was isolated using the GeneJET RNA Purification Kit (#10171080, 2019, ThermoFisher Scientific, USA) according to the manufacturer's instructions. RNA (100 ng) was reverse transcribed into cDNA with RevertAid RT Reverse Transcription Kit (#15255146, 2019, ThermoFisher Scientific) using random hexameric primers (#SO142, 2019, ThermoFisher Scientific) according to the manufacturer's instructions. Quantitative real-time PCR was performed using Maxima SYBR Green qPCR Master Mix (#11340760, 2019, ThermoFisher Scientific, USA) according to manufacturer's instructions and gene expression of rat SOCS3 (fw 5'-TTCTTCACACTGAGCGTCGAGA-3', rev 5'-CTTGAGTACACAGTCAAAGCGGG-3'), c-Fos (fw 5'-AGCATGGGCTCCCCTGTCA-3', rev 5'-GAGACCAGAGTGGGCTGCA-3'), Egr1 (fw 5'-GTATGCTTGCCCTGTTGAGTCC-3', rev 5'-CATGCAGATTCGACACTGGAAG-3'), activity-regulated cytoskeleton-associated protein (Arc)/Arg3.1 (fw 5'-CCCTGCAGCCCAAGTTCAAG-3', rev 5'-GAAGGCTCAGCTGCCTGCTC-3'), and hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) (fw 5'-GCAGACTTTGCTTTCCTTGG-3', rev 5'-TACTGGCCACATCAACAGGA-3') was measured. Quantification of gene expression was calculated as described by Pfaffl et al. (Pfaffl, 2001) using HPRT1 mRNA expression for normalization.

2.6 | Cell cycle analysis

PC12 cells were washed with phosphate-buffered saline (PBS). Subsequently, cells were fixed in 70% ethanol for 1 hr at 4°C. After fixation, cells were washed twice with PBS and incubated with 20 µg/ml RNaseA for 10 min. DNA staining was achieved by adding 50 ng/ml propidium iodide and DNA content was analysed using FACS Canto II (BD Biosciences, USA). Cell cycle analysis was done with FlowJo™ v10.6.2 software.

2.7 | Proliferation and viability assay

Both, viable cell count and viability of PC12 cells were determined using the trypan blue method-based cell counter system Vi-CELL XR (Beckman Coulter LifeSciences). Viable cell count was determined by counting trypan blue negative cells. Total cell count was obtained by

counting trypan blue negative as well as positive cells. Viability was calculated by viable to total cell count ratio.

2.8 | Confocal laser scanning microscopy

PC12 cells were stimulated as indicated and fixed with 2.5% PFA for 20 min at 21°C Room Temperature. For detection of the endogenous GR, cells were permeabilized in 90% methanol for 15 min and stained with an antibody specific for the GR and a secondary anti-rabbit antibody coupled to Alexa647 (#A27040, 2018 ThermoFisher Scientific). Additionally, Hoechst 33342 (#B2261, 2020, Merck, Germany) was added for nuclear DNA staining. Samples were imaged using a Zeiss LSM710 confocal microscope. Quantification of the nuclear localization of Alexa647-stained GR was performed by determining the relative ratio of intensity of the fluorophore Alexa647 in the nucleus and in the cytoplasm. Nuclear fraction (% of nuclear) was calculated as the nuclear localized fluorophore intensity divided by the overall intensity of the fluorophore in the cytoplasm and the nucleus, multiplied by 100.

2.9 | Data analysis and study design

Data of western Blot and qRT-PCR experiments were additionally normalized applying the optimal alignment method described by Degasperi et al. (Degasperi et al., 2014). Optimal alignment is achieved by scaling the data by a scaling factor for each replicate resulting in minimal distance between data across replicates. For this, data were transferred to the MATLAB™ environment (MATLAB™ 2020a) and the normalization was performed with a custom-written script.

Statistical analyses were performed with IBM SPSS Statistics for Windows, version 26 (IBM Corp., USA). Data were tested for normal distribution with the Shapiro-Wilk test. In case of non-normal distributed data, nonparametric tests such as Mann–Whitney-U (for single comparison) and Kruskal–Wallis (followed by post hoc Dunn–Bonferroni for multiple comparisons) were applied. For normal distributed data, parametric tests such as *t*-test (for single comparison) and ANOVA (followed by post hoc Tukey for multiple comparisons) were performed. Data were not tested for outliers. No data were excluded. The designations n.s. = non-significant, * $p < .05$, ** $p < .01$, *** $p < .001$ denote p-values for the measured differences. All experiments contained a minimum of three replicates ($n = 3$). No randomization methods were used or blinding was performed for this study. No pre-determined sample size calculation was applied and the study was exploratory.

2.10 | Ethical statement

No ethical approval was required for this study.

2.11 | Pre-registration

This study was not pre-registered.

3 | RESULTS

3.1 | IL-6 signalling induces neurite outgrowth and reduces proliferation

The ability of IL-6 to induce neurite outgrowth of PC12 cells has been shown previously (Ihara et al., 1997; März et al., 1997; Satoh et al., 1988). Non-differentiated PC12 cells do respond scarcely to IL-6 due to low expression of the IL-6 receptor (IL-6R, gp80) (März et al., 1996, 1997). Cells slightly expressing IL-6R are stimulated physiologically with a complex of IL-6 and the agonistic soluble IL-6R (sIL-6R). sIL-6R arises from shedding of membrane-bound IL-6R or by alternative splicing from IL-6R pre-mRNA and is found at high concentration in serum (Garbers et al., 2012). Hence, using a fusion protein called Hyper-IL-6 (Hy-IL-6), consisting of IL-6 fused to the soluble IL-6R, resembles physiological initiation of IL-6 signal transduction (Chebath et al., 1997). Stimulation of PC12 cells with Hy-IL-6 for 14 days resulted in neurite outgrowth (Figure 1a). Neurites of PC12 cells treated with Hy-IL-6 were significantly longer than neurites of untreated cells (Figure 1b). In general, in cytokine-induced cell fate decisions, proliferation is reduced to promote differentiation. Hence, we studied whether IL-6 signalling affects cell cycle progression. Equal numbers of PC12 cells were seeded and incubated in the absence or presence of Hy-IL-6. After 14 days of cultivation, cell viability and cell counts were determined. Treatment with Hy-IL-6 did not affect PC12 cell viability (Figure 1c). Of note, cell cultures supplemented with Hy-IL-6 showed decreased cell counts in comparison to untreated cell cultures (Figure 1d), indicating that IL-6 signalling counteracts PC12 cell proliferation. Furthermore, cell cycle analyses revealed slight G2 and S to G0/G1 phase transition induced by Hy-IL-6 (Figure 1e). From these results, we conclude that IL-6 signalling reduces PC12 cell proliferation by retaining cells in the G0/G1 phase to allow cell differentiation and neurite outgrowth.

3.2 | Neurite outgrowth, induced by IL-6 signalling, depends on MAPK pathway activation

Expression of constitutive active MAPK demonstrated that MAPK pathway activation is crucial for neuritogenesis in PC12 cells (Cowley et al., 1994). To investigate the impact of IL-6 signalling-induced MAPK pathway activation on neurite outgrowth, we studied Hy-IL-6-induced neurite outgrowth of PC12 cells in the presence of the Mek-inhibitor U0126. PC12 cells were treated with Hy-IL-6 and either with U0126 or the solvent control DMSO for 7 days. Hy-IL-6-induced neurite outgrowth was significantly impaired in the presence of U0126 (Figure 2a and b), demonstrating that neurite outgrowth, induced by IL-6 signalling, depends on the activation of the MAPK pathway.

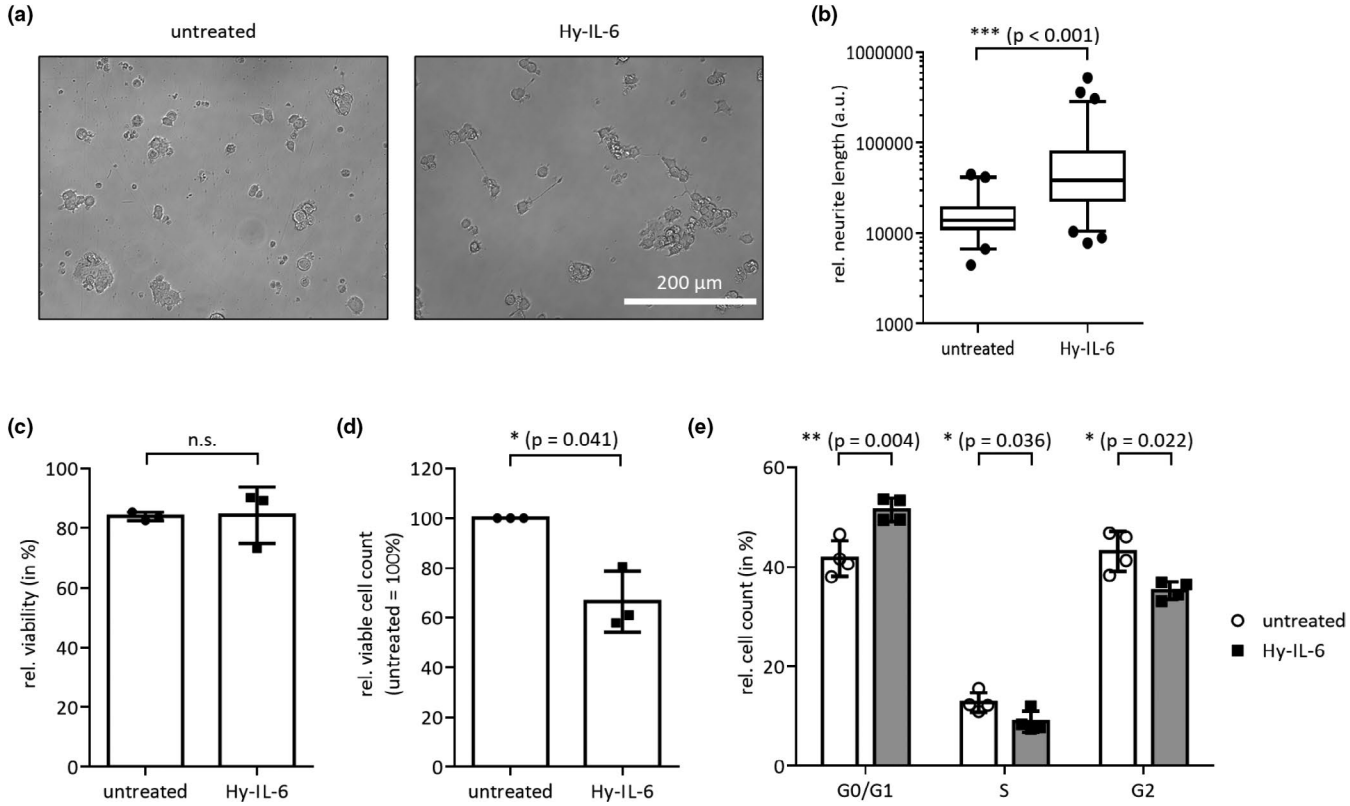

FIGURE 1 Interleukin-6 (IL-6) signalling induces neurite outgrowth in PC12 cells. (a) PC12 cells were seeded on poly-L-lysin-coated dishes and cultivated in phenol red-free RPMI-1640 containing 1% fetal bovine serum (FBS) for 24 hr. On the following day, cells were treated with 100 ng/ml Hyper-IL-6 (Hy-IL-6) or were left untreated. Medium was changed after 7 days and treatment with Hy-IL-6 was continued. After 14 days, neurite outgrowth was analysed with phase-contrast microscopy. Representative results of $n = 3$ independent cell cultures are shown. (b) Neurite lengths were quantified. Distribution of 30 data points in each sample is displayed with Box plots; boxes enclose 25th and 75th percentiles of each distribution and are bisected by the median of neurite length; whiskers indicate 5th and 95th percentiles; data points outside the 5th and 95th percentiles are shown. Mann–Whitney-U-test: n.s. = non-significant, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. (c) Total and viable cells were counted and relative cell viability was calculated by the ratio of viable and total cells in %. (d) Relative viable cell count was calculated by the viable cell count compared to the untreated control (= 100%). (e) Flow cytometric based analysis of permeabilized and propidium iodide stained PC12 cells for cell-cycle distribution. Relative cell count per cell cycle is given in % of total cell count. Data in (c–e) are given as mean of $n = 3$ independent cell culture preparations ± standard deviation (*SD*). Student's *t*-test: n.s. = non-significant, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

3.3 | IL-6 signalling induces expression of c-Fos and Egr1 in a MAPK pathway-dependent manner

Neurite outgrowth is known to depend on cytokine-induced expression of immediately early genes (IEGs) coding for proteins such as early growth response protein 1 (Egr1) or c-Fos (Adams et al., 2017; Gil et al., 2004). Thus, we were interested in whether Hy-IL-6 induces the transcription of these IEGs. Indeed, PC12 cells stimulated with 100 ng/ml Hy-IL-6 for 60 min showed induced expression of c-Fos-mRNA and Egr1-mRNA (Figure 2c and d). Moreover stimulation with 200 ng/ml Hy-IL-6 did not lead to increased Hy-IL-6-induced mRNA expression indicating for sufficient IEG induction upon 100 ng/ml Hy-IL-6 (Fig. S1). Next, we analysed whether activation of the MAPK cascade is involved in c-Fos and Egr1 mRNA expression, induced by IL-6 signalling. Thus, PC12 cells were treated with U0126 30 min prior to Hy-IL-6 stimulation. As expected, Mek-inhibition abolished Hy-IL-6-induced Erk1/2 phosphorylation but did not affect Hy-IL-6-induced STAT3 phosphorylation, the latter indicating proper initiation of IL-6-induced signalling by Hy-IL-6 (Figure 2e, for quantification, see 2nd and 3rd panel). Furthermore, inhibition of Mek-activity attenuated the Hy-IL-6-induced mRNA expression of c-Fos and Egr1 (Figures 2c, b, 3rd column), demonstrating that IL-6 signalling induces gene induction of c-Fos and Erg1 in a MAPK pathway-dependent manner.

3.4 | Glucocorticoids attenuate neurite outgrowth induced by IL-6 signalling

Stress-induced glucocorticoids and synthetic glucocorticoids, such as Dex impact neural development and neurite outgrowth and thereby contribute to neuropathologies (Gould & Tanapat, 1999; Dos Santos et al., 2019) (for review, see (Odaka et al., 2017)). Hence, we were interested in whether Dex also affects IL-6 signalling-induced neuritogenesis. We treated PC12 cells with Hy-IL-6, Dex, or Hy-IL-6 in combination with Dex for 14 days and subsequently

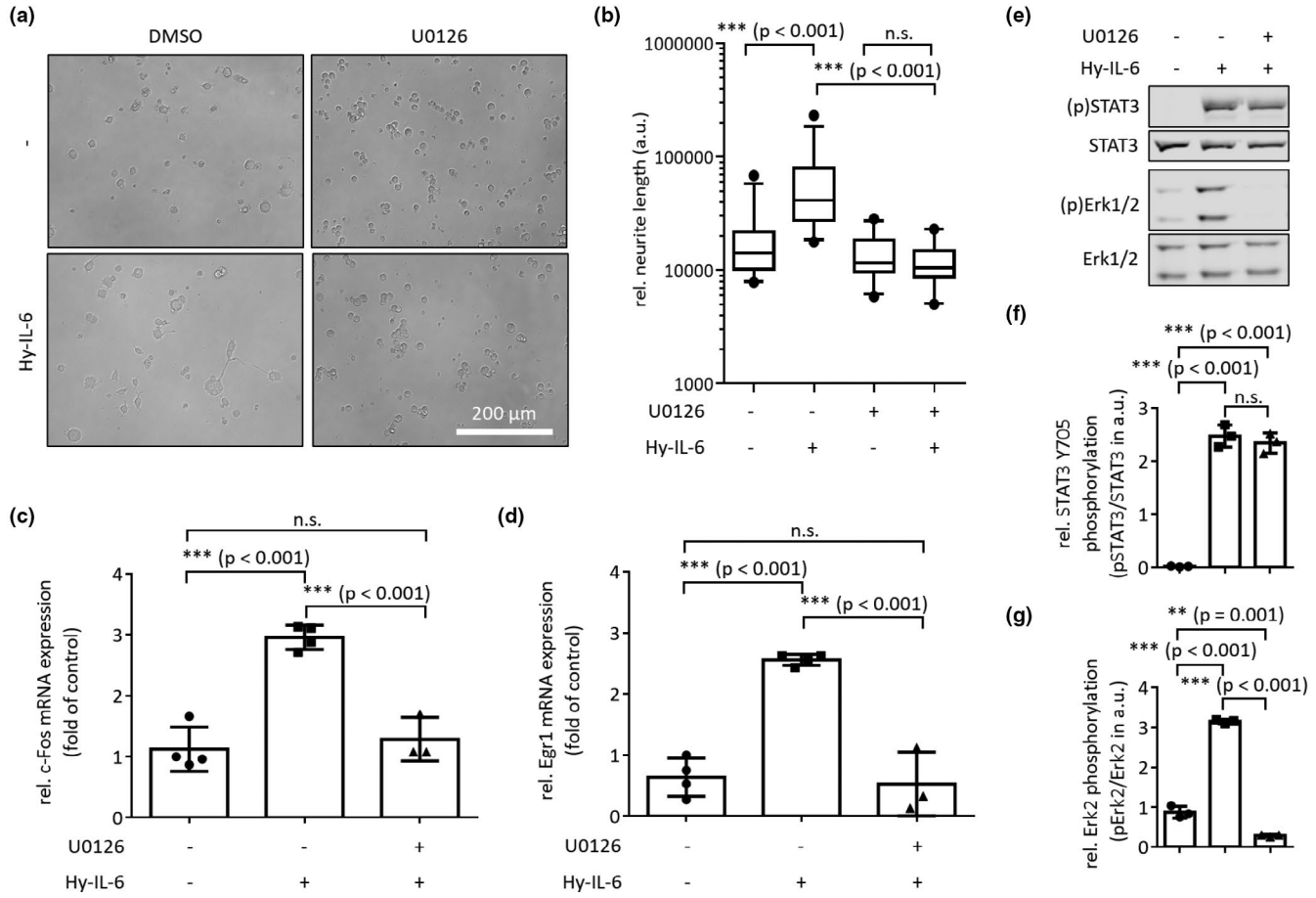

FIGURE 2 IL-6 signalling-induced neurite outgrowth and c-Fos and Egr1 mRNA expression depend on MAPK pathway activation. (a) PC12 cells were seeded on poly-L-lysin-coated dishes and cultivated in phenol red-free RPMI-1640 containing 1% FBS for 24 hr. On the following day, cells were treated with the solvent control DMSO in the absence or presence of 10 µM U0126 and with 100 ng/ml Hy-IL-6 as indicated. After 7 days, neurite outgrowth was analysed with phase-contrast microscopy. Representative results of $n = 3$ independent cell cultures are shown. (b) Neurite lengths were quantified. Distribution of 30 data points in each sample is displayed with Box plots; boxes enclose 25th and 75th percentiles of each distribution and are bisected by the median of neurite length; whiskers indicate 5th and 95th percentiles; data points outside the 5th and 95th percentiles are shown. Kruskal–Wallis test with post hoc Dunn–Bonferroni comparisons: n.s. = non-significant, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. (c–g) PC12 cells were seeded on poly-L-lysin-coated dishes and cultivated in phenol red-free RPMI-1640 containing 1% FBS for 24 hr. On the following day, cells were pre-treated with 10 µM U0126 or its solvent control DMSO for 30 min prior to stimulation with 100 ng/ml Hy-IL-6 for 60 min. (c and d) Total mRNA was extracted and subjected to qRT-PCR analysis to monitor c-Fos (c) and Egr1 (d) mRNA expression. The expression of c-Fos and Egr1 mRNA was normalized to hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) mRNA expression. Relative expression of mRNA is given in fold of control mRNA expression. Data are given as mean of four independent cell culture preparations ± *SD*. One-way ANOVA test with post hoc Tukey comparisons: n.s. = non-significant, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. (e) For Western blot analysis of proteins, cell lysates were prepared and proteins separated by SDS-PAGE. After Western blotting, membranes were stained for phosphorylated signal transducer and activator of transcription 3 ((p)STAT3), total STAT3, phosphorylated extracellular signal regulated kinase ((p)Erk1/2) and total Erk1/2. Representative results of $n = 3$ independent cell culture preparations are shown. For quantification of STAT3 Y705 phosphorylation and Erk2 phosphorylation, signal intensities of (p)STAT3 and total STAT3 as well as (p)Erk2 and total Erk2 were analysed via densitometry. The diagrams show the ratio of (p)STAT3 to total STAT3 (f) and (p)Erk2 to total Erk2 (g). Data are given as mean of three independent cell culture preparations ± *SD*. One-Way ANOVA test with post hoc Tukey comparisons: n.s. = non-significant, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

analysed neurite outgrowth. Dex treatment did not induce neurite outgrowth whereas Hy-IL-6 induced neurite outgrowth as demonstrated in the experiments before (Figure 3a and b). Interestingly, Hy-IL-6-induced neurite outgrowth was impaired in the presence of Dex, showing that glucocorticoids attenuate neurite outgrowth induced by IL-6 signalling.

3.5 | Glucocorticoids reduce Expression of IEGs induced by IL-6 signalling

To determine a potential mechanism explaining impaired neuritogenesis by glucocorticoids, we examined the expression of c-Fos and Egr1 induced by IL-6 signalling in the presence of Dex.

Hy-IL-6 induced mRNA expression of c-Fos, Egr1, and SOCS3 already 60 min after administration of Hy-IL-6 (Figure 3c–e). Dex treatment alone did not induce expression of these mRNAs. Interestingly, Hy-IL-6-induced mRNA expression of c-Fos and Egr1 was reduced in the presence of Dex (Figure 3c and d and Fig. S2a and b). In contrast, expression of SOCS3-mRNA was not affected by Dex treatment (Figure 3e and Fig. S2c). These results demonstrate that IEG gene induction induced by IL-6 signalling is counteracted by glucocorticoids in a gene-specific manner.

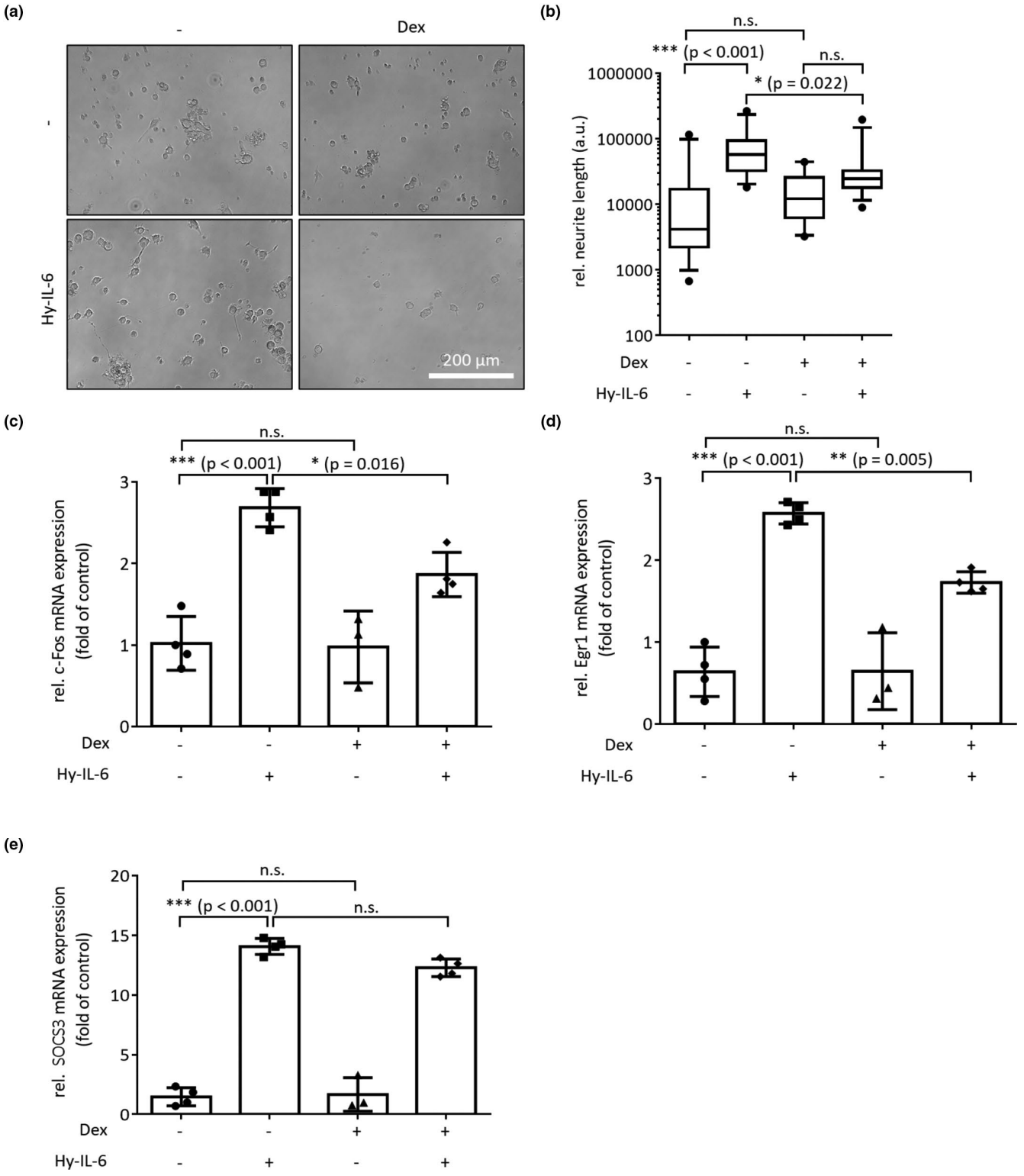

FIGURE 3 Glucocorticoids attenuate neurite outgrowth and reduce immediate early gene mRNA expression induced by IL-6 signalling. (a) PC12 cells were seeded on poly-L-lysin-coated dishes and cultivated in phenol red-free RPMI-1640 containing 1% FBS for 24 hr. On the following day, cells were treated with 1 μM Dexamethasone (Dex) and with 100 ng/ml Hy-IL-6, as indicated. Medium was changed after 7 days and Dex, as well as Hy-IL-6, treatment was continued. After 14 days, neurite outgrowth was analysed by phase-contrast microscopy. Representative results of $n = 3$ independent cell cultures are shown. (b) Neurite lengths were quantified. Distribution of 30 data points in each sample is displayed with Box plots; boxes enclose 25th and 75th percentiles of each distribution and are bisected by the median of neurite length; whiskers indicate 5th and 95th percentiles; data points outside the 5th and 95th percentiles are shown. Kruskal–Wallis test with post hoc Dunn-Bonferroni comparisons: n.s. = non-significant, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. (c–e) PC12 cells were seeded on poly-L-lysin-coated dishes and cultivated in phenol red-free RPMI-1640 containing 1% FBS for 24 hr. On the following day, cells were pre-treated with 1 μM Dex for 30 min prior to stimulation with 100 ng/ml Hy-IL-6 for 60 min as indicated. Total mRNA was extracted and subjected to qRT-PCR analysis to monitor c-Fos (c), Egr1 (d) and suppressor of cytokine signalling 3 (SOCS3) (e) mRNA expression. The expression of c-Fos, Egr1 and SOCS3 mRNA was normalized to HPRT1 mRNA expression. Relative expression of mRNA is given in fold of control mRNA expression. Data are given as mean of four independent cell culture preparations ± *SD*. One-Way ANOVA test with post hoc Tukey comparisons: n.s. = non-significant, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

3.6 | Glucocorticoids neither affect Erk nor STAT3 phosphorylation induced by IL-6 signalling

Neurite outgrowth, induced by IL-6 signalling, was highly dependent on MAPK pathway activity (Figure 2a and b). MAPK signalling did also facilitate gene induction of c-Fos and Egr1 (Figure 2c and d). Both, neurite outgrowth (Figure 3a and b) and gene induction of c-Fos and Egr1 (Figure 3c and d) are reduced by glucocorticoids. Hence, we were interested in whether glucocorticoids interfere with IL-6 signalling-induced Erk1/2 activation thus attenuating IEG expression and neurogenesis. Erk and STAT3 phosphorylation were obvious in PC12 cells stimulated with Hy-IL-6 (Figure 4a). Pretreatment with Dex neither altered phosphorylation of Erk nor STAT3 (Figure 4a, for quantification, see Figure 4B and C). These data show that glucocorticoids do not affect neurite outgrowth by interfering with Erk phosphorylation induced by IL-6 signalling.

3.7 | Neurite outgrowth diminished by Dex can be rescued by the GR antagonist RU-486

As Dex did not interfere with IL-6 signalling-induced activation of MAPK (Figure 4), we hypothesized that glucocorticoids act directly on transcriptional activation of c-Fos and Egr1 encoding genes. Thus, we first made sure that Dex induces nuclear translocation of the GR in PC12 cells. Treatment of PC12 cells with Dex for 90 min-induced nuclear localization of the GR in the presence and absence of Hy-IL-6 (Figure 5a). Interestingly, stimulation of PC12 cells with Hy-IL-6 alone also induced translocation of the GR into the nucleus. However, this effect did not appear to the same extent as Dex-induced nuclear translocation of the GR (Figure 5b). These results demonstrate Dex-induced GR translocation into the nucleus of PC12 cells and let us speculate that glucocorticoids act on IL-6 signalling-induced neurite outgrowth through the transcriptional activity of the GR. To clarify whether the transcriptional activity of the GR accounts for reduced neurite outgrowth upon glucocorticoid treatment, we utilized the GR antagonist RU-486 (also known as mifepristone). As shown before, Hy-IL-6 induced neuritogenesis (Figure 5b and c). PC12 cells treated with RU-486 alone showed no neurite outgrowth whereas, in combination with Hy-IL-6, neurite outgrowth of PC12 cells was induced to the same extent as with Hy-IL-6 treatment alone. While Dex attenuated Hy-IL-6-induced neurite outgrowth, Dex treatment in combination with RU-486 did not impair Hy-IL-6-induced neuritogenesis. Taken together, Dex impairs neurite outgrowth induced by IL-6 signalling. The antagonist RU-486 does not interfere with neurite outgrowth induced by IL-6 signalling. However, RU-486 restores IL-6 signalling-induced neuritogenesis in the presence of Dex.

3.8 | RU-486 restored c-Fos and Egr1 mRNA expression attenuated by Dex

The GR antagonist RU-486 did not reduce neurite outgrowth induced by IL-6 signalling, but rather restored Dex-related reduction of neuritogenesis. Hence, we were interested in whether the Dex-related reduction of c-Fos-mRNA and Egr1-mRNA expression can be restored by RU-486. PC12 cells were treated with RU-486 and/or Dex 30 min prior to stimulation with Hy-IL-6 for 60 min. Hy-IL-6 alone induced mRNA expression of c-Fos and Egr1 which was reduced in the presence of Dex. RU-486 alone did not affect mRNA expression of c-Fos or Egr1. Furthermore, RU-486 restored IL-6 signalling-induced mRNA expression in the presence of Dex (Figure 6a and b). Hy-IL-6-induced SOCS3-mRNA expression neither was affected by Dex nor by RU-486 (Figure 6c). These results show that Dex negatively acts on transcriptional activation of IEGs in a gene-specific manner which can be restored with the GR antagonist RU-486.

3.9 | Altered response towards IL-6 and glucocorticoid signalling in differentiated PC12 cells

As differentiation leads to reprogramming of cellular processes, we were interested in whether long-term IL-6 treatment reprograms PC12 cells regarding sensitivity towards IL-6 and glucocorticoid signalling. Hence, mRNA expression of genes coding for proteins such as c-Fos, Egr1 and Arc/Arg3.1 related to neural development (Gallo et al., 2018) was monitored after 14 days Hy-IL-6 treatment in the presence or absence of Dex. Interestingly, PC12 cells treated with Hy-IL-6 for 14 days showed reduced expression of Egr1 and

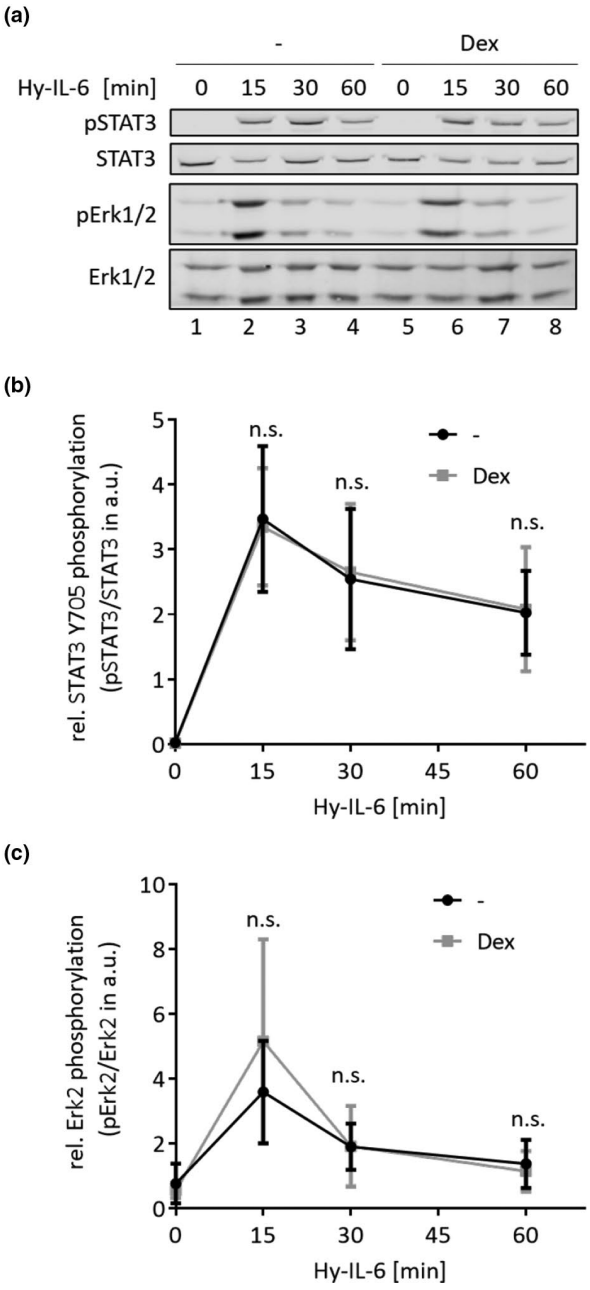

FIGURE 4 Glucocorticoids neither affect Erk1/2 nor STAT3 phosphorylation induced by IL-6 signalling. (a) PC12 cells were seeded on poly-L-lysin-coated dishes and cultivated in phenol red-free RPMI-1640 containing 1% FBS for 24 hr. On the following day, cells were pre-treated with 1 μM Dex for 30 min prior to stimulation with 100 ng/ml Hy-IL-6 for times indicated. Subsequently, cell lysates were prepared and proteins separated by SDS-PAGE. After western blotting, membranes were stained for (p)STAT3, total STAT3, (p)Erk1/2, and total Erk1/2. Representative results of $n = 6$ independent cell culture preparations are shown. For quantification of STAT3 Y705 phosphorylation and Erk2 phosphorylation, signal intensities of (p)STAT3 and total STAT3 as well as (p)Erk2 and total Erk2 were determined via densitometry. The diagrams show the ratio of (p)STAT3 to total STAT3 (b) and (p) Erk2 to total Erk2 (c). Data are given as mean of six independent cell culture preparations ± *SD*. Two-Way ANOVA test: n.s. = non-significant, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

Arc/Arg3.1 mRNA compared to untreated cells (Figure 7b and c). In contrast, c-Fos-mRNA expression was not significantly altered upon long-term Hy-IL-6 treatment (Figure 7a). Hy-IL-6-induced SOCS3 mRNA expression was still observed after 14 days post-Hy-IL-6-administration (Figure 7d). As SOCS3 mRNA is very short-lived (Ehlting et al., 2007), this observation indicates ongoing IL-6 signalling even in long-term treatment.

Additionally, we also examined the long-term effect of Dex on mRNA expression of c-Fos, Egr1, Arc/Arg3.1, and SOCS3. Long-term Dex treatment significantly reduced basal Arg/Arc3.1 mRNA expression (Figure 7c). In contrast, Dex long-term treatment did not affect mRNA expression of c-Fos and Egr1 in PC12 cells (Figure 7a and b). Moreover neither basal nor Hy-IL-6-induced SOCS3 mRNA expression was affected by Dex (Figure 7d). Furthermore, Hy-IL-6 in combination with Dex had no additional effect on c-Fos, Egr1 and Arc/Arg3.1 mRNA expression compared to Hy-IL-6 alone (Figure 7b and c). Long-term Dex treatment in the absence or presence of Hy-IL-6 was accompanied with accumulation of the GR in the nucleus (Fig. S3). This indicates for ongoing glucocorticoid-induced signalling upon long-term Dex administration after 14 days and therefore glucocorticoid-induced signalling might account for the Dex-dependent reduction in Arc/Arg3.1 mRNA expression.

Taken together, these results show that long-term Hy-IL-6 and Dex treatment result in gene expression profiles of IEGs that differ from short-term Hy-IL-6 and Dex-treated PC12 cells (compare Figures 3c and d and 7a and b). Since long-term Hy-IL-6 treatment induces differentiation of PC12 cells, this might lead to reprogramming of PC12 cells concerning expression of specific IEGs in response to IL-6 and glucocorticoids.

4 | DISCUSSION

IL-6 impacts on neurogenesis and neural development in early-life and adulthood (for reviews, see Erta et al., 2012; Heese, 2017)). However, the mechanisms, underlying IL-6-induced neural differentiation remain elusive, so far. To illuminate molecular mechanisms accounting for IL-6-induced neural differentiation we utilized PC12 cells, an established model for neural differentiation. PC12 cells express the IL-6R but scarcely respond to IL-6 (Wu & Bradshaw, 1996). März et al. showed that IL-6 alone induces expression of genes accounting for differentiation of PC12 cells (März et al., 1996). However, the amount of gene products induced by IL-6 is not sufficient to induce neuritogenesis of PC12 cells. This could be due to low IL-6R expression level causing insufficient ligand:receptor complex formation, thus preventing neurite outgrowth of PC12 cells (März et al., 1996). In contrast to IL-6 alone, the potential of the IL-6:sIL-6R complex, as well as the designer protein Hy-IL-6, to induce neuritogenesis of PC12 cells has been reported, previously (Ihara et al., 1997; März et al., 1997; Satoh et al., 1988; Valerio et al., 2002; Wu & Bradshaw, 1996).

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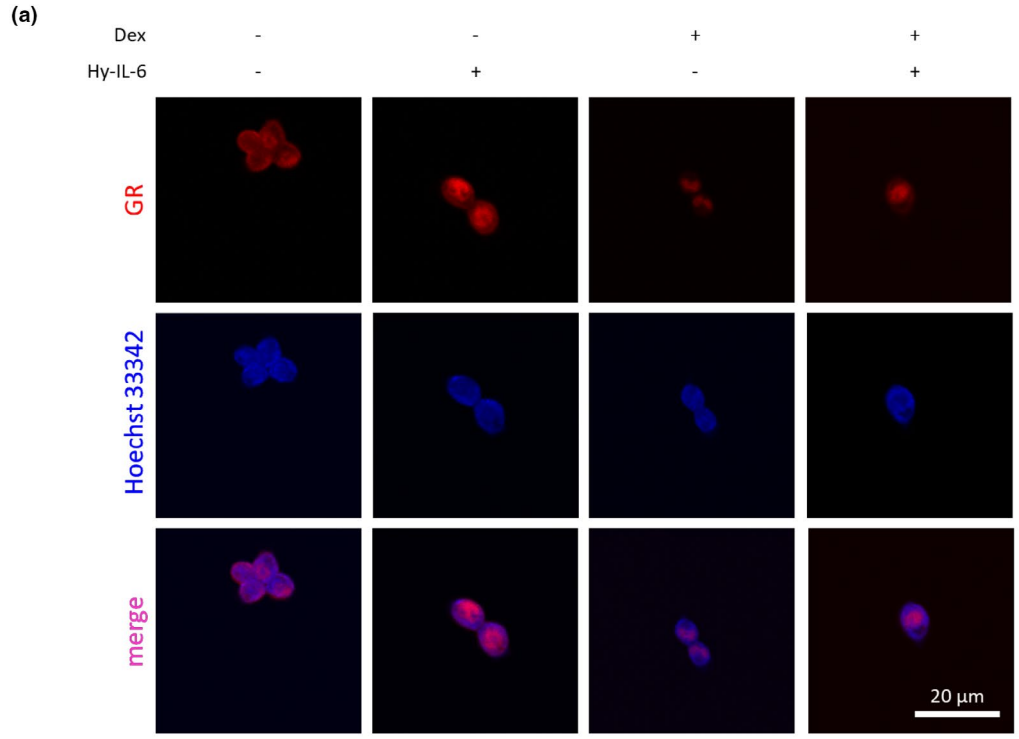

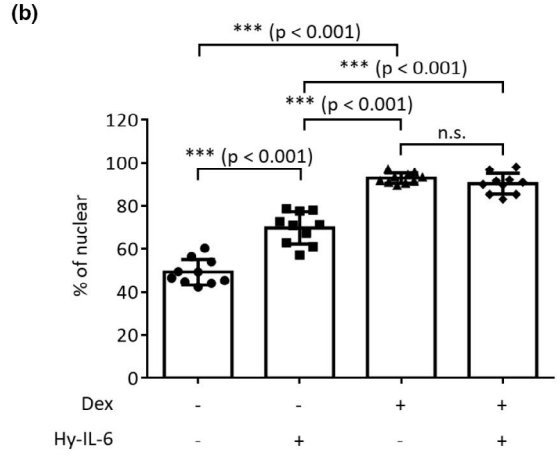

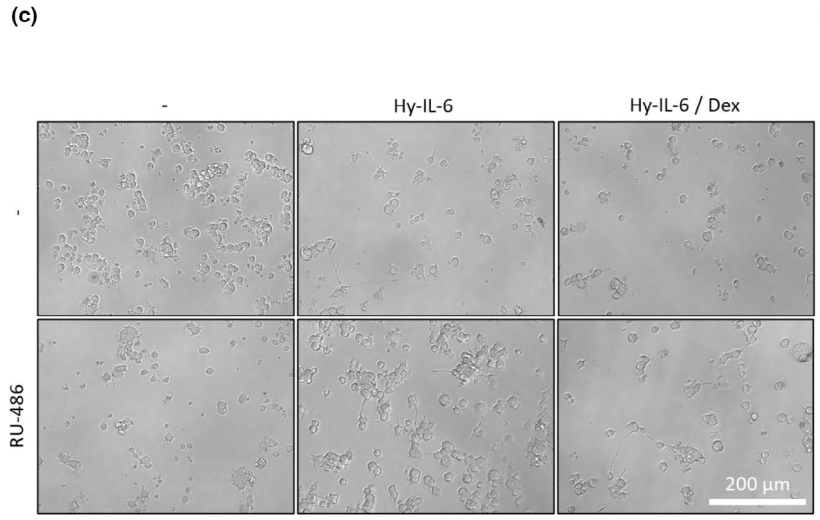

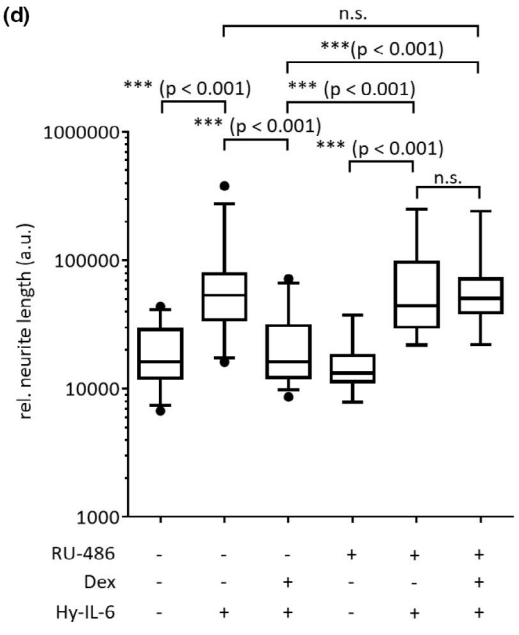

FIGURE 5 Neurite outgrowth diminished by glucocorticoids can be rescued by the GR antagonist RU-486. (a) PC12 cells were seeded on poly-L-lysin-coated glass cover slides and cultivated in phenol red-free RPMI-1640 containing 1% FBS for 24 hr. On the following day, cells were pre-treated with 1 μM Dex for 30 min prior to stimulation with 100 ng/ml Hy-IL-6 for 60 min. Subsequently, cells were fixed, permeabilized and stained for endogenous glucocorticoid receptor (GR). Additionally, cells were stained with Hoechst 33342. Imaging was performed with a confocal laser scanning microscope. Representative results of $n = 3$ independent cell culture preparations are shown. (b) Nuclear fraction (% of nuclear) of the GR was quantified from three independent cell culture preparations. Data are given as mean of 10 cells ± *SD*. One-Way ANOVA test with post hoc Tukey comparisons: n.s. = non-significant, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. (c) PC12 cells were seeded on poly-L-lysin-coated dishes and cultivated in phenol red-free RPMI-1640 containing 1% FBS for 24 hr. On the following day, cells were treated with 1 μM Dex and/or 1 μM RU-486 and with 100 ng/ml Hy-IL-6, as indicated. Medium was changed after 7 days and Dex, RU-486, as well as Hy-IL-6 treatment was continued. After 14 days, neurite outgrowth was analysed by phase-contrast microscopy. Representative results of $n = 3$ independent cell cultures are shown. (d) Neurite lengths were quantified. Distribution of 30 data points in each sample is displayed with Box plots; boxes enclose 25th and 75th percentiles of each distribution and are bisected by the median; whiskers indicate 5th and 95th percentiles; data points outside the 5th and 95th percentiles are shown. Kruskal–Wallis-test with post hoc Dunn–Bonferroni comparisons: n.s. = non-significant, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

Here, we could confirm that IL-6 signalling induces neurite outgrowth of PC12 cells (Figure 1). Significant neurite outgrowth was observed after 7 to 14 days of Hy-IL-6 treatment (Figure 1 and Figure 2). The cell count of PC12 cells was lower for cells treated with Hy-IL-6 than for untreated cells (Figure 1c), whereas cell viability was not affected (Figure 1d). Hence, the lower cell count can be explained by reduced proliferation of PC12 cells, treated with Hy-IL-6. Moreover Hy-IL-6 affects the cell cycle progression of PC12 cells and enhances the number of cells in the G0/G1 phase (Figure 1e). These results indicate that IL-6 signalling prolongs the G0/G1 phase of PC12 cells. *In vitro* and *in vivo* studies demonstrated prolonged G1 phase as prerequisite for neural differentiation (Calegari & Huttner, 2003; Dobashi et al., 2000). Taken together, the prolonged G0/G1 phase and unaltered cell viability of PC12 cells after 14 days of Hy-IL-6 treatment support the observation of IL-6 signalling-induced neural differentiation.

Additionally, we showed that IL-6 signalling induced c-Fos and Egr1 mRNA expression (Figure 2c and d). c-Fos and Egr1 are IEGs and their expression is essential for physiological brain development and plasticity (for review, see (Gallo et al., 2018)). Blocking expression or function of either c-Fos or Egr1 impairs NGF-induced neuritogenesis of PC12 cells (Gil et al., 2004; Levkovitz & Baraban, 2002). Here, we show that Mek-activity is crucial for expression of these IEGs in response to IL-6 signalling. IL-6-induced MAPK pathway activation depends on the phosphorylation of tyrosine 759 within the cytoplasmic part of gp130, the signal-transducing subunit of the entire IL-6R complex. MAPK-activation is central for the downstream activation of the transcription factor Elk-1 (Lehmann et al., 2003). Phosphorylation and activation of Elk-1 lead to the expression of c-Fos (Janknecht et al., 1993). März et al. observed that the c-Fos protein amount is enhanced in response to IL-6 in PC12 cells (März et al., 1997). Hence, IL-6 triggers c-Fos expression at both, mRNA as well as protein level in neuron-like cells. The c-Fos protein is part of the transcription factor complex AP-1 (Angel & Karin, 1991; Chiu et al., 1988). Reduced c-Fos expression or knockout in mice reduces synaptic plasticity in the hippocampus indicating the central role of c-Fos and the AP-1 complex in neural development and brain functions. However, the underlying molecular mechanisms of c-Fos to regulate synaptic plasticity and brain functions are yet not well understood. In addition to c-Fos, we also observed IL-6 signalling-induced Egr1 mRNA expression in PC12 cells as previously reported by Kunz et al. (Kunz et al., 2009). In neural development and function, Egr1 expression is essential. Attenuated Egr1 expression accounts for several neuropsychiatric disorders (for review, see (Duclot & Kabbaj, 2017)). Nonetheless, the diverse and complex functions of Egr1 in neural development remain elusive. Although, it became clear that Egr1 is involved in neural development by regulating the neural proteasome (James et al., 2005, 2006). Furthermore, Egr1 contributes to dendritic spine remodelling and the growth of synaptic connections, thus is involved in structural plasticity (Lai et al., 2012; Miniaci et al., 2008) (for review, see (Lamprecht & LeDoux, 2004)). Davis et al. demonstrated that Erk-induced hyperphosphorylation of cAMP response element-binding protein (CREB) and Elk-1 trigger Egr1 expression in the hippocampus, particularly in the dentate gyrus (Davis et al., 2000). Thus, Erk-dependent Elk-1 activation might be crucial for c-Fos and Egr1 expression induced by IL-6 signalling.

Consistent with reduced expression of c-Fos and Egr1 upon Mek inhibition, the neurite outgrowth of PC12 cells was abolished in the presence of the Mek-inhibitor (Figure 2c and d). Thus, we conclude that IL-6 signalling-induced neuritogenesis strongly depends on the activity of the Mek-Erk pathway. Similarly, NGF-induced neurite outgrowth of PC12 cells also depends on Erk activity (Cowley et al., 1994; Pang et al., 1995). Furthermore, *in vivo* studies in mice treated with Mek-inhibitors reveal the necessity of Mek-Erk pathway activation for the physiological outgrowth of dendritic spines (Goldin & Segal, 2003; Wu et al., 2001). Taken together, the sensitivity of c-Fos and Egr1 gene expression and neurite outgrowth to Mek inhibition suggests that the activation of the Mek-Erk pathway significantly influences physiological neural differentiation.

Neural development and physiological brain function are impaired by acute and chronic stress. Stress initiates the HPA axis and thereby induces the release of glucocorticoids such as cortisol. High and sustained glucocorticoid levels at early-life or adulthood enhance the risk for the development of neuropathologies (Majcher-Maslanka et al., 2018; Oitzl et al., 1997) (for reviews, see Gould & Tanapat, 1999; McEwen, Bowles, et al., 2015)). Moreover administration of synthetic glucocorticoids to prevent pregnancy complications, such as pre-term birth or neonatal respiratory distress syndrome by promoting fetal lung maturation (Crowther et al. 2015; Health 1994)

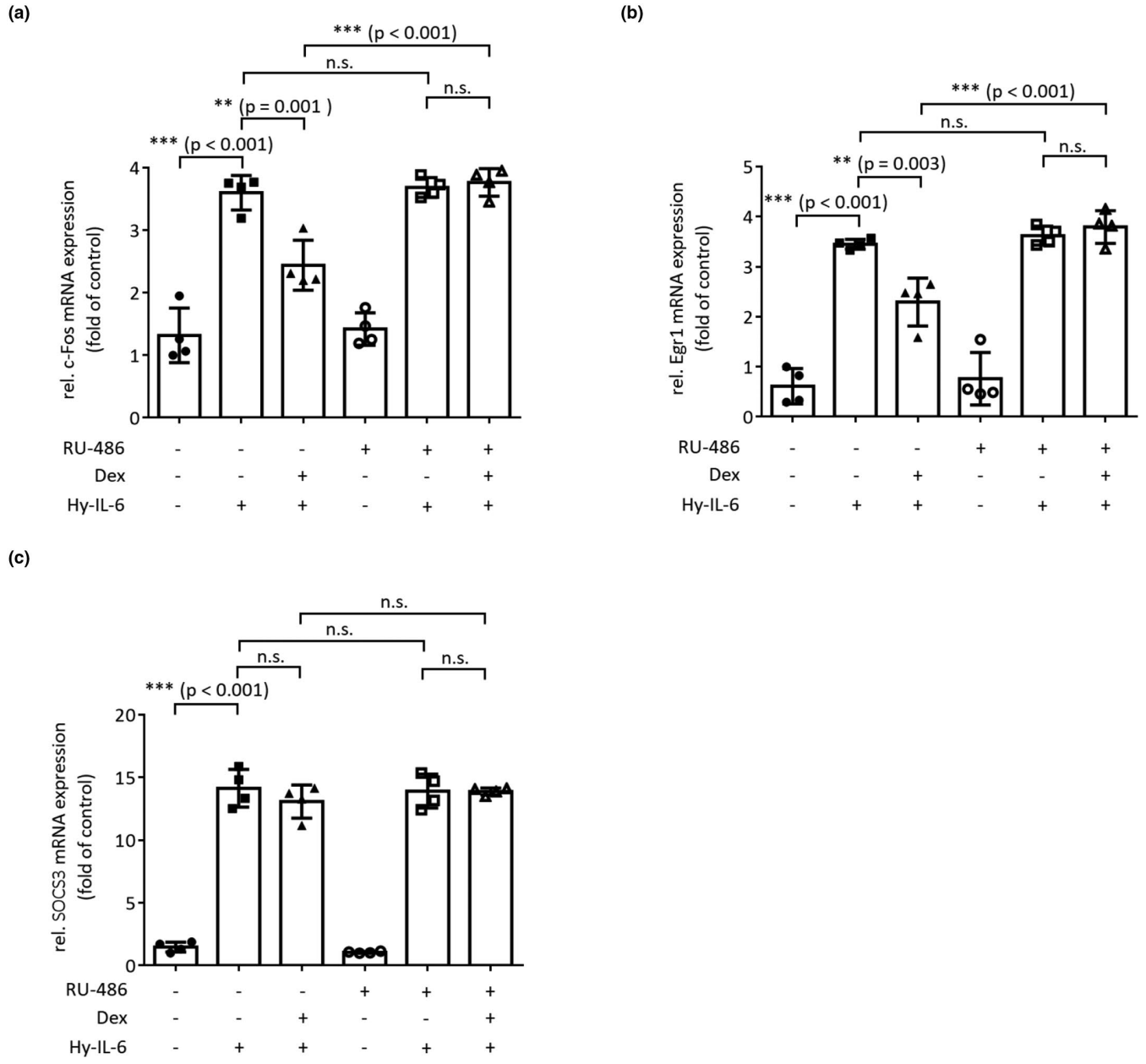

FIGURE 6 RU-486 rescues c-Fos and Egr1 mRNA expression diminished by Dex. PC12 cells were seeded on poly-L-lysin-coated dishes and cultivated in phenol red-free RPMI-1640 containing 1% FBS for 24 hr. On the following day, cells were pre-treated with 1 μM Dex and/or 1 μM RU-486 for 30 min prior to stimulation with 100 ng/ml Hy-IL-6 for 60 min. Total mRNA was extracted and subjected to qRT-PCR analysis to monitor c-Fos (a), Egr1 (b), and SOCS3 (c) mRNA expression. The expression of c-Fos, Egr1, and SOCS3 mRNA was normalized to HPRT1 mRNA expression. Relative expression of mRNA is given in fold of control mRNA expression. Data are given as mean of four independent cell culture preparations ± *SD*. One-way ANOVA test with post hoc Tukey comparisons: n.s. = non-significant, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

(for review, see (Haram et al., 2017)), is associated with neuropsychiatric disorders like depression or schizophrenia in the adult offspring (Davis et al., 2013; Thompson et al., 2001) (for reviews, see Celano et al., 2011; Hunter et al., 2016)). Additionally, Dex treatment in adulthood to suppress immune activation in rheumatoid arthritis or multiple sclerosis (Cohen et al., 1960; McFarland, 1969) impairs brain functions such as learning and memory (Dong et al., 2018; Machhor et al., 2004; Dos Santos et al., 2019). Therefore, we were interested in whether the glucocorticoid Dex has also the ability to impair IL-6-induced IEG expression and neurite outgrowth. As discussed above, IEG expression and neurite outgrowth are essential processes for neural development and brain function. Indeed, PC12 cells treated with Dex showed impaired neurite outgrowth in response to IL-6 signalling (Figure 3a and b). Terada et al. showed that Dexamethasone also attenuates NGF-induced neurite outgrowth and reduces NGF-induced Erk-phosphorylation (Terada et al., 2014). However, reduced Erk-phosphorylation in response to Dex was not observed in PC12 cells treated with Hy-IL-6 and Dex (Figure 4).

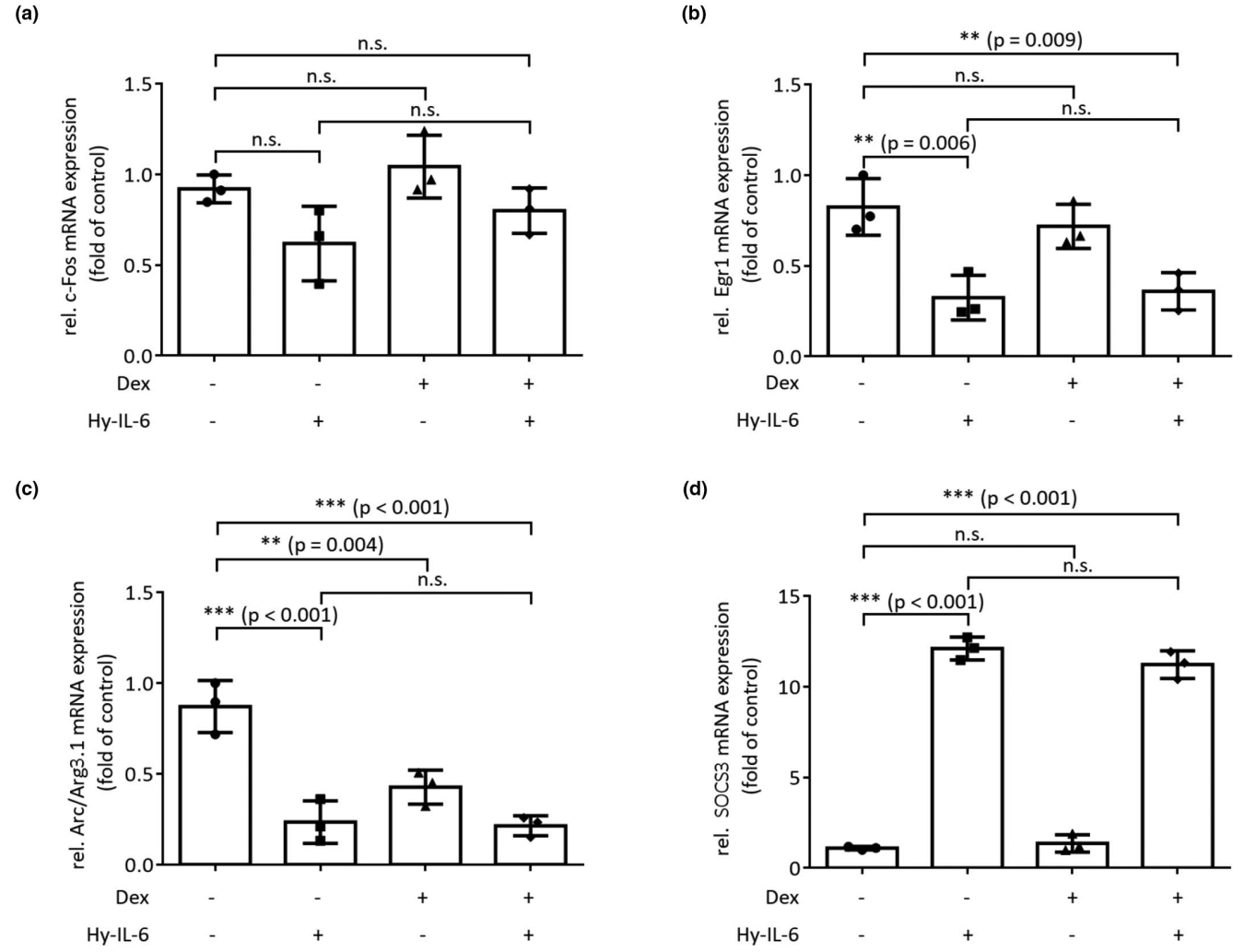

FIGURE 7 Altered response towards IL-6 and glucocorticoid signalling in differentiated PC12 cells. PC12 cells were seeded on poly-L-lysin coated dishes and cultivated in phenol red-free RPMI-1640 containing 1% FBS for 24 hr. On the following day, cells were treated with 1 μM Dexamethasone and with 100 ng/ml Hy-IL-6. Medium was changed after 7 days and Dex, as well as Hy-IL-6 treatment was continued. After 14 days, total mRNA was extracted and subjected to qRT-PCR analysis to monitor c-Fos (a), Egr1 (b), activity-regulated cytoskeleton-associated protein (Arc)/Arg3.1 (c) and SOCS3 (d) mRNA expression. The expression of c-Fos, Egr1, Arc/Arg3.1, and SOCS3 mRNA was normalized to HPRT1 mRNA expression. Relative expression of mRNA is given in fold of control mRNA expression. Data are given as mean of three independent cell culture preparations ± *SD*. One-way ANOVA test with post hoc Tukey comparisons: n.s. = non-significant, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

Nonetheless, the expression of c-Fos and Egr1 induced by IL-6 signalling is reduced by Dex in PC12 cells (Figure 3c and d and Fig. S2a). März et al. showed decreased expression of gp130 in cells treated with Dex (März et al., 1996), which might serve as a plausible explanation for reduced IL-6-induced signalling and subsequent attenuated gene expression. However, it is more likely that Dex impairs IEG expression downstream from receptor and Erk activation because gp130-dependent STAT3 and Erk phosphorylation were unaltered upon Dex treatment (Figure 4). The nuclear localization of the GR in PC12 cells after short-term Dex treatment (Figure 5a and b) indicates Dex-induced GR activation. Once bound to the glucocorticoid responsive element (GRE) in DNA, the GR elicits different modes of transcriptional regulation. On the one hand, GR acts through transactivation, leading to gene expression (for review, see (Flower, 1988)). On the other hand, GR facilitates transrepression of gene expression (Xavier et al., 2016). Alternatively, the activated GR could directly bind to transcription factors, thereby repressing gene expression independent from GREs in target genes (for review, see (Timmermans et al., 2019)). Thus, it is conceivable that the reduction of c-Fos and Egr1 expression by Dex might be due to GR-mediated transrepression. We could show that the GR antagonist RU-486 neither did affect IL-6 signalling-induced IEG expression nor neurite outgrowth (Figures 5c and 6a,b and d). Noteworthy, upon Dex treatment, RU-486 rescues both, IEG expression and neurite outgrowth induced by IL-6 signalling. RU-486 interferes with glucocorticoid-induced activation of the GR abrogating both, transactivation and transrepression (for review, see (Mao et al., 1992)). Taken together, attenuated IL-6 signalling-induced neural differentiation upon Dex treatment might be due to transcriptional repression of c-Fos and Egr1 expression by the activated GR (Figure 8). However, the

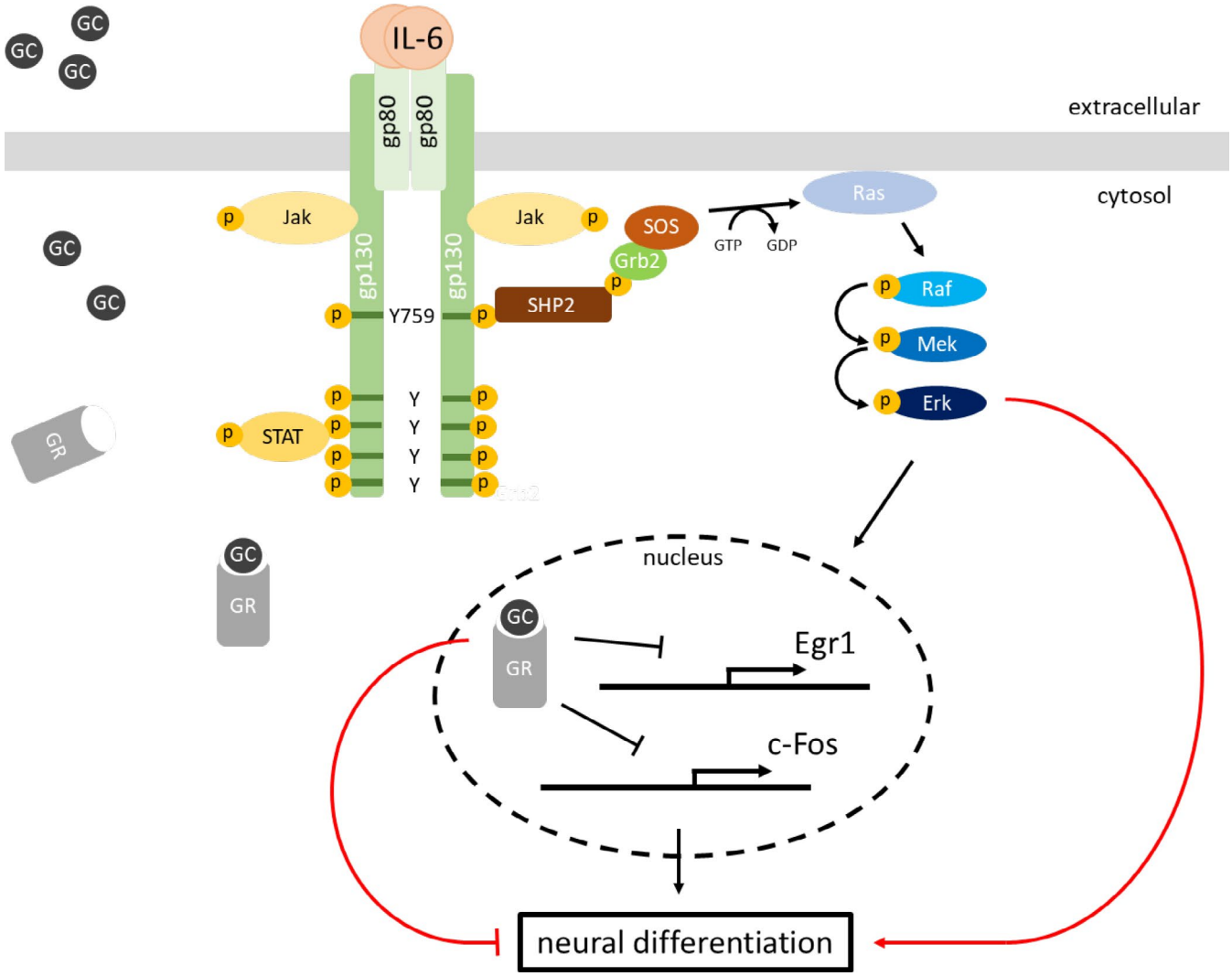

FIGURE 8 Glucocorticoids interfere with IL-6-signalling-induced IEG expression and neuritogenesis. IL-6-induced gp130 receptor activation leads to SHP2/Grb2/SOS complex recruitment and subsequent Ras/Raf/Mek/Erk pathway activation. The activation of the Erk pathway is crucial for IEG expression of c-Fos and Egr1. Both these IEGs are well-characterized in mediating neurite outgrowth and neural differentiation. Stress-induced glucocorticoids (GC) bind the cytosolic glucocorticoid receptor (GR). Nuclear translocation of the activated GC-GR complex is the prerequisite for its function in transcriptional regulation. The GC-GR complex interferes with Egr1 and c-Fos expression induced by IL-6-signaling. Hence, glucocorticoids and stress, respectively, may abrogate IL-6-induced neural differentiation

specific molecular mechanism underlying Dex-associated repression of IL-6 signalling-induced c-Fos and Egr1 gene transcription in PC12 cells remains elusive. Therefore, the multiple ways in which GR-dependent repression of IL-6 signalling-induced IEG expression may occur need to be fully elucidated in future studies to better understand the effects of glucocorticoids on IL-6 signalling-dependent differentiation processes.

Arc/Arg3.1 is another IEG family member and subcellular localization of Arc/Arg3.1 protein in the synapse is crucial for α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) trafficking (Chowdhury et al., 2006). Thus, Arc/Arg3.1 is involved in the regulation of long-term potentiation (LTP) and generation of synapses, important processes in facilitating learning and memory (McIntyre et al., 2005) (for review, see (Minatohara et al., 2015)). Long-term Dex treatment decreased basal Arc/Arg3.1 mRNA expression (Figure 7c). Interestingly, Arc/Arg3.1, as well as Egr1 mRNA expression, were also reduced in PC12 cells which have been differentiated in the presence of IL-6 signalling for 14 days. Thus, we conclude that early expression of Egr1 induced by IL-6 signalling is important for neurite outgrowth and PC12 cell differentiation, whereas long-term IL-6 signalling attenuates Egr1 expression. All these observations indicate for a time-dependent orchestration of IL-6-induced neural development with a beneficial early IEG activation facilitating neural differentiation, whereas long-term IL-6 exposure decreases IEG expression. Interestingly, long-lasting and elevated IL-6 signalling, as is the case in chronic inflammation, correlates with neuropathologies (Sakic et al., 2001; Sukoff Rizzo et al., 2012; Voorhees et al., 2013) (for review, see (Erta et al., 2012)). Hence, it would be interesting to elucidate the time-dependent orchestration of IL-6-affected neural development in future studies.

Short-term treatment with Dex and Hy-IL-6 resulted in reduced IL-6 signalling-induced c-Fos and Egr1 mRNA expression compared to Hy-IL-6 treatment alone (Figure 3c and d). In contrast, long-term treatment with Hy-IL-6 reduces basal expression of these genes. Dex in combination with Hy-IL-6 did not further reduce mRNA expression compared to Hy-IL-6 treatment alone (Figure 7a and b). These observations indicate that Dex and Hy-IL-6 do not act in concert on the reduction of mRNA expression upon long-term treatment. Since the GR was predominantly localized in the nucleus upon long-term treatment with Dex (Fig. S3), the activated GR is proposed to repress mRNA expression even upon long-term stimulation. However, this Dex effect might be concealed by the IL-6-dependent repression of c-Fos, Egr1, and Arc/Arg3.1 mRNA expression upon long-term treatment (Figure 7).

With this study, we provide evidence that glucocorticoids attenuate IL-6 signalling-induced c-Fos and Egr1 mRNA expression (Figure 3c and d) and diminished Arc/Arg3.1 mRNA expression upon long-term treatment (Figure 7c) which was associated with reduced neurite outgrowth (Figure 3a and b). Interestingly, Dex treatment and chronic stress impair physiological neural development and are also associated with several neuropathologies such as depression (Dong et al., 2018; Ieraci et al., 2016; Okada et al., 2015; Dos Santos et al., 2019). Post-mortem analysed brains from patients suffering from depressive disorders show less complex dendritic trees on neurons and a reduced volume of specific brain regions (Arnone et al., 2012; Saylam et al., 2006). These effects are associated with reduced expression of Arc/Arg3.1 and Egr1 in major depressive disorder (MDD) patients and animal models, showing depressive-like behaviour. In contrast, it was shown that acute early-life stress challenges are associated with an induction of Egr1 and Arc/Arg3.1 expression (Xie et al., 2013). The induction is mediated by epigenetic histone modifications presumably induced by high glucocorticoid levels during acute stress exposure and thus might serve as a specific adaptation process to acute stress. Worth mentioning, we observed that Dexamethasone acts in a dose-dependent manner on IL-6 signalling-induced c-Fos expression (Fig. S2a) indicating for dose-dependent effects of glucocorticoids on neural development. Hence, the impact of the duration of stress exposure and GC concentration on gene expression in neural development should be elucidated in more detail in future studies. Nonetheless, all these observations indicate the impact of Arc/Arg3.1 and Egr1 in neural development and neuropsychiatric disorders (Hodges & McCormick, 2015; Ieraci et al., 2016; Lee et al., 2012; Matsumoto et al., 2012; Okada et al., 2014; Ons et al., 2010). Since reduced Egr1 and Arc/Arg3.1 expression in PC12 cells after long-term IL-6 or glucocorticoid treatment are in line with observations made after chronic inflammation and stress, PC12 cells represent a potent model to elucidate the molecular mechanisms in stress- and inflammation-induced dysregulated neural development.

Stress-triggered GR activation during early-life brain development as well as in hippocampal adult neurogenesis leads to disturbed neural development (Anacker et al., 2013; Davis et al., 2013) (for review, see (Gould & Tanapat, 1999)). Synthetic glucocorticoids—such as Dex—negatively affect early-life as well as adult neurogenesis and plasticity (Dong et al., 2018; Machhor et al., 2004; Dos Santos et al., 2019). Here, we showed that Dex impairs IL-6-induced IEG expression and neural differentiation of PC12 cells. Our data suggest that stress, as well as Dexamethasone treatment, reduce the expression of IEGs such as c-Fos, Egr1, and Arc/Arg3.1 and thereby diminish neural development indicating the sensitivity of Interleukin-6-dependent neural differentiation towards stress or glucocorticoid treatment. These results contribute to a deeper understanding of the molecular mechanisms of stress, glucocorticoids, and IL-6 on neural differentiation. Hence, it is worth elucidating the impact of glucocorticoids on IL-6 signalling-induced cell fate decisions in more physiological models of neural differentiation in future studies.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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