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A transcriptome comparison of time-matched developing human, mouse and rat neural progenitor cells reveals human uniqueness

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ABSTRACT

It is widely accepted that human brain development has unique features that cannot be represented by rodents. Obvious reasons are the evolutionary distance and divergent physiology. This might lead to false predictions when rodents are used for safety or pharmacological efficacy studies. For a better translation of animal-based research to the human situation, human in vitro systems might be useful. In this study, we characterize developing neural progenitor cells from prenatal human and time-matched rat and mouse brains by analyzing the changes in their transcriptome profile during neural differentiation. Moreover, we identify hub molecules that regulate neurodevelopmental processes like migration and differentiation. Consequences of modulation of three of those hubs on these processes were studied in a species-specific context. We found that although the gene expression profiles of the three species largely differ qualitatively and quantitatively, they cluster in similar GO terms like cell migration, gliogenesis, neurogenesis or development of multicellular organism. Pharmacological modulation of the identified hub molecules triggered species-specific cellular responses. This study underlines the importance of understanding species differences on the molecular level and advocates the use of human based in vitro models for pharmacological and toxicological research.

1. Introduction

Pharmacological research as well as drug safety testing have mainly been based on studies in laboratory animals. Besides ethical concerns and high resource needs with regards to time and money, laboratory animals do frequently fail to predict beneficial or adverse compound effects for humans leading to high attrition rates when moving from preclinical research to clinical drug applications (Arrowsmith and Miller, 2013; Waring et al., 2015), e.g. rodents only correctly identified 43% out of 150 pharmaceuticals known to be toxic in man (Olson et al., 2000). Also, an analysis of systematic reviews published in the "Scopus" database on the human clinical or toxicological utility of animal experiments revealed that in only 10% of the reviews animal models were significantly useful (Knight, 2007). Several reasons were identified to be responsible for this unsatisfactory translation from animals to humans. For one, poor performance quality and reporting of animal studies impedes prediction (Hartung, 2009; Kilkenny et al., 2010; Knight, 2007; Leist and Hartung, 2013; van der Worp et al., 2010). Moreover, species differences can hamper predictive value of animal in vivo studies (Gold et al., 2005; Knight, 2007; Leist and Hartung, 2013; Seok et al., 2013). Such limited predictions might result in high attrition rates during the drug development process causing economic damage and lack of treatment (De Keyser et al., 1999; Perel et al., 2007; Seok et al., 2013; Waring et al., 2015). Incorrect information on human safety or toxicity of substances is equally severe as it might result in serious human health effects as experienced with thalidomide or TGN1412, which did not show animal toxicity in the species tested but caused serious toxicities in humans (Attarwala, 2010; Miller and Stromland, 1999). While performance quality and reporting of animal studies can be improved e.g. by introducing more stringent quality

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criteria (Dirnagl and Fisher, 2012), species differences in human vs. animal physiology and/or pathology cannot rapidly be overcome using in vivo whole animal studies.

To improve "human relevance" in safety and efficacy studies, one can make use of human cell-based in vitro methods. Although such methods lack pharmaco-/toxicokinetics of the whole organism (absorption, distribution, metabolism, excretion), they are thought to maintain their pharmaco-/toxicodynamics of the target cell in vitro. There are many examples for species-specific differences in the molecular equipment of cells that can determine compound action (Jenkins et al., 2012; Oberheim et al., 2009; Perreault et al., 2013; Seok et al., 2013; Strasser et al., 2013).

We have previously shown that such specificities in toxicodynamics across species are also maintained in primary, time-matched neurospheres from humans, mice and/or rats in vitro (Baumann et al., 2015; Dach et al., 2017; Gassmann et al., 2010). Three dimensional (3D) neurospheres consist of neural progenitor cells (NPC) that allow assessment of compound-specific effects on NPC proliferation, radial migration, differentiation into neurons, astrocytes and oligodendrocytes as well as neuronal migration and neurite outgrowth (Barenys et al., 2016; Baumann et al., 2015, 2014; Dach et al., 2017; Gassmann et al., 2012; Schmuck et al., 2016). Moreover, modes of action of substances can be studied with this organoid cell culture method (Barenys et al., 2016; Dach et al., 2017; Moors et al., 2007; Schreiber et al., 2010). Due to these attributes, this "Neurosphere Assay" is thought to be a valuable part of an alternative testing battery for developmental neurotoxicity (DNT) evaluation (Fritsche, 2016; Bal-Price et al., 2018).

This transcriptome-based study was designed to understand the species-specific nature of immature brain cells, analyze the pathways underlying the neurodevelopmental functions that can be studied using the "Neurosphere Assay" (Baumann et al., 2015; Bal-Price et al., 2018) and compare pathway functions between the three species: human, mouse and rat. These species were chosen due to human relevance, presence of transgenic animals and regulatory usage, respectively. Specific marker gene expression analyses identified human-specific traits in the human compared to rodent NPC. Gene Ontology (GO) term clustering (Bindea et al., 2009) and subsequent protein-protein interaction enrichment analyses (Bindea et al., 2013; Szklarczyk et al., 2015) were used to identify the functional pathways driving NPC functions computationally. These were then validated by functional studies in vitro using respective model compounds across the three species. The functionally validated microarray data defines the biological application domain of the "Neurosphere Assay" and identifies species specificities in signaling relevant for neurodevelopmental functions.

2. Material and methods

2.1. Chemicals

Bone morphogenetic protein 2 (BMP2) was purchased from R&D Systems (#355-BM; Wiesbaden, Germany). A stock solution (2.5 µg/mL) was prepared in B27 medium. *N*-[*N*-(3,5-Difluorophenacetyl)-L-alanyl]-*S*-phenylglycine t-butyl ester (DAPT) and Epidermal Growth factor receptor (EGFR) -inhibitor PD153035 were purchased from Sigma-Aldrich (#D5942, #SML0564; Taufkirchen, Germany). For DAPT and PD153035 stock solutions of 40 mM and 10 mM were prepared in dimethyl sulfoxide (DMSO; Carl Roth GmbH; Karlsruhe, Germany), respectively. Working solutions were prepared in N2 with 0, 0.1, 0.13 or 0.25% DMSO.

2.2. Cell culture

Human neural progenitor cells [hNPC, male, gestational week (GW) 16–18] isolated from whole brain were purchased from Lonza Verviers SPRL (CAT. # PT-2599; LOT NO.:0000339988 and 0000277692; Verviers, Belgium). Rodent neural progenitor cells [post-natal day (PND)1] were prepared as described previously for rat (Baumann et al., 2014) and mouse (Dach et al., 2017). The time point for rodent sample preparation (PND1) was selected based on a comparative algorithm across species (Workman et al., 2013). According to this algorithm, brain developmental processes in GW 16–18 in human NPC are most similar to processes observed in rodent brain development at PND1 (www.translatingtime.org). Human and rodent NPC were cultured as neurospheres in proliferation medium consisting of DMEM (Life Technologies, Darmstadt, Germany) and Hams F12 (Life Technologies; 3:1) supplement with 2% B27 (Life Technologies), 1% penicillin and streptomycin (Pan-Biotech, Aidenbach, Germany), 20 ng/mL epidermal growth factor (EGF, Life Technologies), 20 ng/mL recombinant human fibroblast growth factor (FGF, R&D systems) for hNPC and mNPC and 10 ng/mL recombinant rat FGF (R&D systems) for rNPC. The culture was maintained at 37 °C with 5% CO2. The culture was fed every two to three days by replacing half the medium with fresh medium and passaged every week by mechanical chopping of the spheres with a tissue chopper (Mcllwain Tissue Chopper, Vibratome). To initiate differentiation, NPC were plated on poly-D-lysine/laminin (Sigma Aldrich) coated 6 well plates in differentiation medium for 3 and 5 days. The differentiation medium consisted of DMEM (Life Technologies) and Ham F12 (Life Technologies; 3:1) supplement with 1% N2 (Life Technologies) and 1% penicillin and streptomycin (Pan Biotech). For differentiation of mNPC 1% fetal calf serum (FCS; Biochrome, Berlin, Germany) was added to the culture medium.

2.3. The "Neurosphere Assay"

To analyze pathway modulation on a functional level we quantified cell migration, as well as neuronal- and oligodendrocyte differentiation in differentiating neural progenitor cells after treatment with the pharmacological modulators BMP2, DAPT and PD153035. Therefore, spheres with a diameter of 0.3 mm were plated on poly-D-lysine/laminin (Sigma Aldrich) coated 8 chamber slides in differentiation medium for 3 and 5 days. On day 3 half the medium was replaced with freshly prepared medium. Migration was analysed 24 h and 72 h after plating. After 3 or 5 days spheres were fixed in 4% paraformaldehyde for 30 min at 37 °C and stained for neurons (βIII-tubulin positive cells) or oligodendrocytes (O4 positive cells). Cell viability was analysed with the Alamar Blue assay (CellTitier-Blue assay, Promega, Mannheim, Germany) in the same chamber/well that was used to analyze the DNT-specific endpoint (Baumann et al., 2014, 2015). Automatic counting of cell numbers in the migration area using the Omnisphero software (Schmuck et al., 2016) was an additional indicator for cell viability. For analyses of astrocyte maturation after BMP2 treatment, hNPC were also stained with an antibody against GFAP after 3 days of differentiation. Astrocyte maturation was quantified as migration distance of radial glia compared to total astrocyte migration distance because an increase in cell maturation increases the amount of mature stellate like astrocytes at the expense of radial glia (Fig. 6j). Apoptotic oligodendrocytes (O4 positive cells with condensed staining around nuclei; Fig. B. 5 l) were counted and normalized to the number of nuclei.

2.4. Generation of RNA samples

For proliferating conditions (0d), 75 neurospheres with a diameter of 0.3 mm were collected for each replicate. For differentiation conditions (3d, 5d) neurospheres were chopped to 0.1 mm and plated at a density of 440 pieces/well in a poly-D-lysine/laminin coated 6 well plate for 3 or 5 days, respectively. RNA was isolated from a total of 54 samples, 6 replicates for each condition (0d, 3d and 5d), and species (h-, r-, and mNPC). RNA isolation was performed with the miRNeasy kit (Qiagen, Hilden, Germany) according to the manufactures protocol. Total RNA was used for microarray analysis and qRT-PCR. Quality of total RNA was analysed with the 2100 bioanalyzer (Agilent

Technologies). In parallel to the generation of RNA samples we performed the "Neurosphere Assay" to control if the cells from the same passage used for RNA analysis proliferate, differentiate and migrate according to our historical controls.

2.5. qRT-PCR analysis

For validation of microarray experiments we performed qRT-PCR of a set of 12–16 genes (Fig. B. 6). Therefore, RNA from the microarray samples harvested on day 0, 3, 5 was transcribed to cDNA using the QuantiTect Rev. Transcription Kit (Qiagen; Hilden, Germany) according to the manufacturer's protocol. Quantitative polymerase chain reaction (qRT-PCR) was performed using the Rotor Gene Q Cycler (Qiagen) with the QuantiFast SYBR Green PCR Kit (Qiagen) according to the manufacturer's protocol. All genes with the respective primer sequences are presented in Table A. 16. Differential expression was calculated with the ddCT method using beta actin for normalization and 0-day samples (proliferating NPC) were used as a reference set to 1.

2.6. Affymetrix arrays

For human NPC synthesis of cDNA and subsequent biotin labelling of cRNA was performed according to the manufacturer's protocol (3′ IVT Plus Kit; Affymetrix, Inc.). Briefly, 100 ng of total RNA each were converted to cDNA, followed by in vitro transcription and biotin labelling of amplified cDNA. After fragmentation, labelled cDNA was hybridized to Affymetrix PrimeView Human Gene Expression Microarrays for 16 h at 45 °C, stained by strepatavidin/phycoerythrin conjugate and scanned as described in the manufacturer's protocol.

For rodent samples synthesis of biotin labelled cDNA was performed according to the manufacturer's protocol (WT Plus Reagent Kit; Affymetrix, Inc.). Briefly, 100 ng of total RNA were converted to cDNA. After amplification by in vitro transcription and 2nd cycle synthesis, cDNA was fragmented and biotin labelled by terminal transferase. Finally, end labelled cDNA was hybridized to Affymetrix Mouse/Rat Gene 2.0 ST Gene Expression Microarrays for 16 h at 45 °C, stained by strepatavidin/phycoerythrin conjugate and scanned as described in the manufacturer's protocol.

2.7. Data analysis and statistics

Data analyses on Affymetrix CEL files was conducted in R'. Probes within each probe set were summarized by Robust multichip average (RMA) after quantile normalization of probe level signal intensities across all samples to reduce inter-array variability. The algorithms where provided by the R packages affy (Gautier et al., 2004) for human samples and oligo (Carvalho and Irizarry, 2010) for mouse and rat samples. Differential gene expression (DEX) was statistically determined by one-way ANOVA followed by Tukey's range test (R Core Team, 2016). The significance threshold was set to $p \leq .01$ (FDR corrected; gene expression analyses). Probes that matched this threshold for no probe set were dismissed. The Affymetrix IDs were translated into gene symbols using a translation table containing all genes matching our Affymetrix IDs. It was built using the biomaRt R package (Durinck et al., 2005). In a first step, a table with the gene symbols and expression data from their corresponding Affymetrix IDs was created. For genes, which matched to more than one Affymetrix ID, the mean of the differential expression per time frame was used. The *p*-values were combined applying Fisher's method (Fisher, 1992).

The Principal Component Analysis (PCA) is based on the significance threshold filtered expression data. Therefore, we worked with 18 data points (6 replicates per time point) per gene per species. Division by its respective median (median for one gene over 18 samples) normalized the expression data of each Affymetrix ID. We chose all genes with identical gene symbol in all species and used the prcomp method from the R stats package (R Core Team, 2016) to perform the PCA. An adjacency matrix was calculated from the expression profiles of the genes over time using the scrime package (Schwender and Fritsch, 2013). Hierarchical clustering was performed by hclust using unweighted pair group agglomeration method with arithmetic mean and cutree (R Core Team, 2016) resulting in 10 distinct clusters. These 10 clusters were manually summarized into the modules M1 – M4 according to their expression profiles over time. To allow a direct species comparison of gene expression independent of differential gene expression, we defined genes as likely to be expressed (present) or likely to be not expressed (absent) based on criteria adapted from Kang et al. (2011). A gene is called present if the median log2 intensity value in 0-day samples is ≥ 6. Genes that do not meet this criterion are defined as absent. The expression profile of single genes in heatmap (Fig. 2g) was prepared with the Multiple Experiment Viewer (MeV 4.9.0; http://mev.tm4.org/).

All data analyses for functional endpoints were performed using GraphPad Prism 6.00 (GraphPad Software, La Jolla California USA, www.graphpad.com). Statistical significance analyses were performed on data normalized to solvent control (except for cell migration, here we used raw data on migration distance) from at least 3 independent experiments by one-way ANOVA followed by Dunnett's multiple comparisons test. The significance cut-off was set to $p \leq .05$.

2.8. Biological process analysis/gene ontological annotation analysis

We used the Cytoscape (version 3.4.0) plug in ClueGO/CluePedia (version 2.2.5; Bindea et al., 2013; Szklarczyk et al., 2015) for overrepresentation analyses of biological process GO clustering and network visualization. The statistical test for overrepresentation analysis was based on a two-sided hypergeometric option with a Bonferroni correction. We chose a *p*-value cut-off of 0.01 and a kappa score of 0.5 for GO clustering with at least two GO terms within one cluster (see parameters for analysis in Table A. 17).

To generate gene interaction networks we used information from the STRING Protein-Protein Interaction database (Szklarczyk et al., 2015; http://string-db.org) within CluePedia, extracted the GO clusters cell migration, gliogenesis and neurogenesis and enriched all genes associated with the selected GO clusters with information on gene-gene or protein-protein interactions (binding, activation, expression and inhibition) with a confidence score of ≥ 0.75. Highly connected genes were identified as those genes showing at least three times (migration, neurogenesis) or two times (gliogenesis) the number of connection compared to the mean number of connection per gene within the respective cluster.

3. Results

3.1. Analyses of changes in NPC mRNA expression profiles across time and species

To determine the changes in molecular equipment of NPC growing as neurospheres during the change from proliferation to differentiation (Baumann et al., 2015), we first analysed mRNA expression profiles of proliferating (0), as well as 3 and 5 days differentiating human, mouse and rat primary neural progenitor cells (hNPC, mNPC, rNPC; Fig. 1) by using microarrays (human PrimeView Array, mouse/rat Gene 2.0 ST Arrays, Affymetrix). Genes were called differentially expressed (DEX) if they were significantly ($p \leq .01$) up- or downregulated by at least 2-fold between any of the three time points (0 vs. 3, 0 vs. 5, or 3 vs. 5 days). In hNPC, mNPC and rNPC a total of 1684, 1979 and 2324 genes, respectively, are DEX across all time points. The number of genes regulated in the first 3 days of differentiation (0 vs. 3) is in the same order of magnitude between the three species (1121 in hNPC, 1196, in mNPC, 1033 rNPC). With continuing NPC differentiation and maturation, more genes are DEX at day 5 (0 vs. 5) with 1531 in hNPC, 1566 in mNPC and 2159 in rNPC. Of those, 971, 849 and 927 genes (> 70%), respectively,

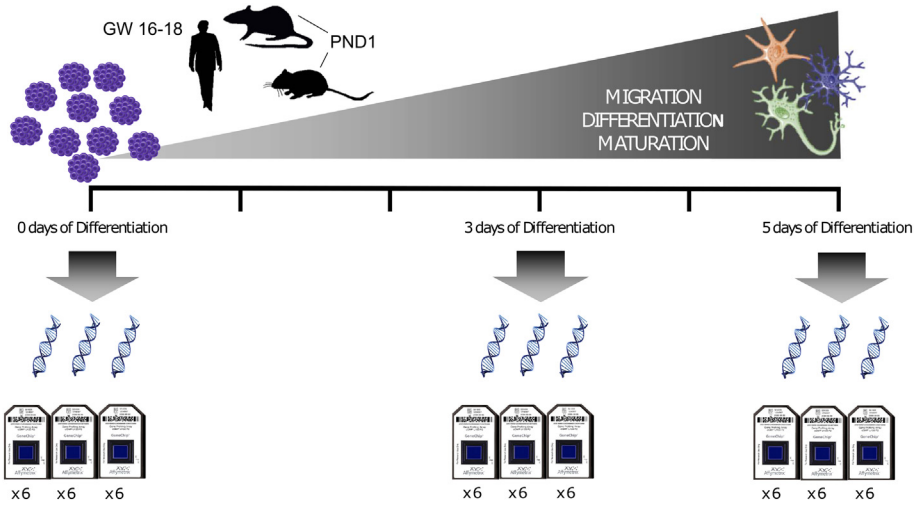

Fig. 1. Experimental set up.
NPC were generated from fetal human brain (GW16–18) or post-natal mouse and rat brain (PND1) and cultivated as floating neurospheres. RNA was isolated from proliferating (0d), as well as three and five days differentiated human, mouse and rat NPC, from 6 replicates per condition and species. Transcriptome analyses from these samples were performed using human PrimeView Array and mouse/rat Gene 2.0 ST Arrays from Affymetrix.

overlap with DEX genes at day 0 vs. 3 (Fig. 2 a-c). In rodent compared to human NPC there is a higher number of DEX genes between 3 and 5 days (43 hNPC, 362 mNPC, 732 rNPC; Fig. 2d) indicating a stronger change in cultures over 5 days in rodent compared to human NPC.

We next determined the number of commonly regulated genes between the total of 1684, 1979 and 2324 DEX genes in hNPC, mNPC and rNPC, respectively, across all time points (0vs3, 0vs5 and 3vs5 days combined). Of these, only 186 (11%) genes share the same gene symbol in all three species (Fig. 2e). This small number of overlapping DEX genes during NPC differentiation can only in part be explained by the dissimilarities of array chips, because of the 1684 DEX genes identified in hNPC 83% (1398) are present on both rodent array chips, yet are not DEX in the rodent cells (data not shown). To compare the magnitude of gene expression changes across the three species in addition to this qualitative difference of gene regulation during NPC differentiation, quantitative differences in magnitude of expression of all regulated genes ($p \leq .01$; 5571) with the same gene symbols across differentiation time and between the three species were evaluated by PCA (Fig. 2f). PC1 (33% explained variation) reveals that for all species 0d samples cluster further from 3d than 3d from 5d samples, pointing towards largest gene expression changes when the cell program switches from NPC proliferation to neural differentiation compared to smaller changes during further maturation of differentiated cells from day 3 to day 5 in culture. For the species comparison, the PCA demonstrates that hNPC have a distinct differentiation dynamic compared to the rodent NPC as visible in the course of expressional changes over time (Fig. 2f, dotted lines). Differences in the differentiation dynamics are also obvious in the expressional changes of genes specific for certain cell types during brain development (Fig. 2g). Obvious examples are the neuronal marker *NRXN1* which is upregulated in hNPC (4.2 and 5.2 fold at 3 and 5 days, respectively), downregulated in mNPC (0.7 and 0.6 fold at 3 and 5 days, respectively) and not present on the microarray chip of rNPC, or the ventricular radial glia marker *ANXA1* which is downregulated in hNPC (0.3 and 0.4 fold at 3 and 5 days, respectively) and upregulated in rodent NPC (13.5 and 3.1 fold in mNPC, 2.6 and 2.6 fold in rNPC at 3 and 5 days, respectively). To allow a direct species comparison of gene expression independent of differential gene expression, we defined genes as likely to be expressed (present) or likely to be not expressed (absent) based on criteria adapted from Kang et al. (2011; only present if median log2 intensity value in 0-day samples is ≥ 6). With this threshold we identified several genes that are likely to be only expressed in human cells (*HS6ST2*, *LRRC3B*, *CALB2* or *ARHGAP11B*; Fig. 2g and Table A. 19). Taken together, the qualitative and quantitative expression data show that primary NPC from human and rodent origins obtain species-specific expression changes when differentiating to neural effector cells with only few DEX genes shared between human and rodent NPC and with some human specific marker genes for NPC development.

For further analyses of gene function behind these expression changes, we generated expression clusters using a hierarchical cluster analysis (HCA) for all DEX genes (Fig. B. 1). We summarized these clusters according to their regulation pattern over time into 4 modules: module 1 (M1) and module 2 (M2) contain genes with the main expression changes, up and down, respectively, within the first 3 days of differentiation and no further regulation from day 3 to day 5 (Fig. 2h). These clusters contain similar numbers of genes across all species (M1: 611, 575, 504; M2: 667, 499, 404, for h-, m- and rNPC, respectively). We hypothesized that these are genes involved in the NPC program change from proliferation to neuronal and glial differentiation and the onset of cellular migration. Summarized in module 3 (M3) and module 4 (M4) are those genes, which are up- and down-regulated, respectively, mainly between day 3 and 5 in vitro. For these clusters, the numbers of genes differ between human and rodent NPC (M3: 191, 547, 887; M4: 215, 358, 428, for h-, m-, rNPC, respectively). These genes are thought to be mainly involved in processes of effector cell maturation and thus the fewer regulated genes in hNPC compared to their rodent counterparts might explain the lack in hNPC-derived neuronal maturation which seems to be enhanced in rNPC-derived neurons (Odawara et al., 2014; Ohara et al., 2015). Mean expression change of the modules M1 and M3 (Fig. 2h) corroborate the observation that mNPC and especially rNPC show stronger expression changes between 0 and 5 days than hNPC (Fig. 2d).

3.2. Overrepresentation analyses (ORA) of gene ontology (GO) biological processes (BP)

To computationally determine biological functions of genes in the temporal expression clusters, we performed ORA for the GO-terms BP using the Cytoscape plugin ClueGO (Bindea et al., 2009). We first analysed the biological functions of genes in clusters M1 and M2, where we expected the largest transcriptional changes due to the switch in cell program from proliferation to differentiation. From the 1278 DEX genes in hNPC, 1074 in mNPC and 908 in rNPC, respective 1070 (83.7%), 904 (84.2%) and 787 (86.7%) were annotated to 155 (hNPC), 427 (mNPC) and 228 (rNPC) GO terms (Table 1; Table A. 1–3). Although developing NPC from humans and rodents have only little overlap in DEX genes (11% of hNPC), 90 of the 155 (58%) GO terms enriched in hNPC were shared with rodent NPC. These GO terms are major BP involved in general organ development like anatomical structure morphogenesis (GO:0009653), organ specific developmental processes like central nervous system development (GO:0007417), or GO terms specific to some major processes of brain development that are assessed by the

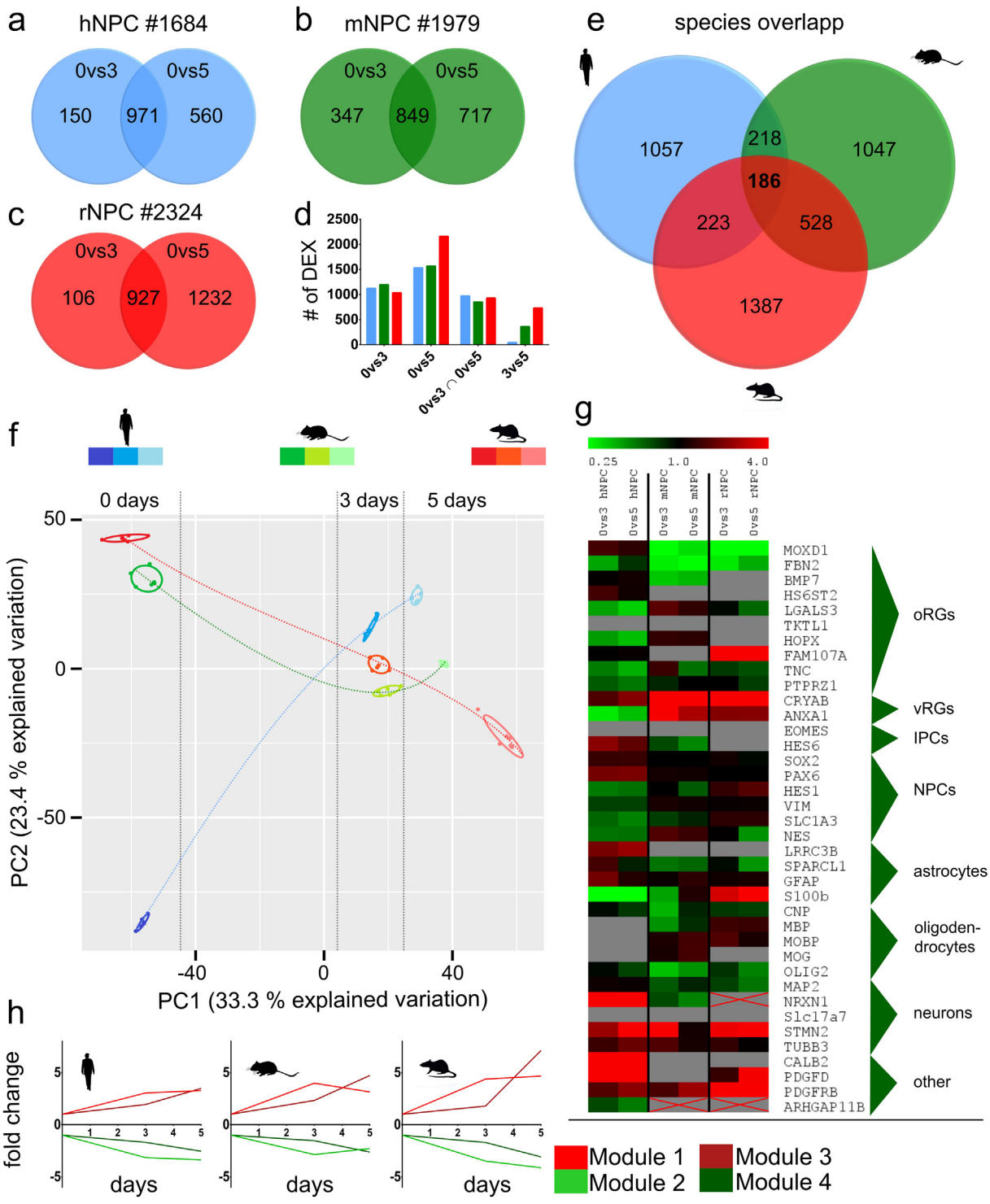

Fig. 2. Qualitative and quantitative comparison of expression profiles across species.
Differential gene expression within each species and between time points was statistically determined by one-way ANOVA followed by Tukey's range test. Genes with $p \leq .01$ and fold change ≥ 2 were called differentially expressed (DEX). (a-c) Overlap of the number of DEX genes between 3 (0vs3) and 5 (0vs5) days of differentiation for human (a), mouse (b) and rat (c) NPC. (d) Comparison of the number of DEX genes for each time point (0vs3, 0vs5, overlap between 0vs3 and 0vs5 and 3vs5) between human (blue), mouse (green) and rat (red) NPC. (e) DEX genes (at any time point) that share the same gene symbol in human (blue), mouse (green), and rat (red) NPC. (f) Principal component analysis (PCA) was performed based on the expression of all significantly regulated ($p \leq .01$) genes that shared the same gene symbol between species (5570) and compares the expression profile over time (0, 3 and 5 days; dark to pale) between species (human, blue; mouse, green; rat, red). (g) Expression profile of single genes associated to specific cell types during neural development and in the CNS of human, mouse and rat NPC. Genes, defined as not present are depicted in grey, genes depicted in grey with a red cross are not on the respective microarray chip. (h) Hierarchical clustering generated 10 distinct expression clusters (Table A. 1), which were further summarized into 4 modules. Data is represented as mean DEX over time of all genes within one module for human mouse and rat NPC. oRG is outer radial glia; vRG is ventricular zone radial glia; IPC is intermediate progenitor cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
GO BP overrepresentation analyses.

Species	Human		Mouse		Rat	
Expression cluster	M1 + 2	M3 + 4	M1 + 2	M3 + 4	M1 + 2	M3 + 4
# genes	1278	404	1074	906	908	1417
# Genes Annotated	1070	345	904	761	787	1058
# GO terms	**155**	**79**	**427**	**110**	**228**	**61**
Shared GO terms	# (% human) of GO terms					
Expression cluster	*M1 + M2*			*M3 + M4*		
Human-Mouse	122 (78)			33 (42)		
Human-Rat	98 (63)			0		
All species	90 (58)			0		
Mouse-Rat	181			45		

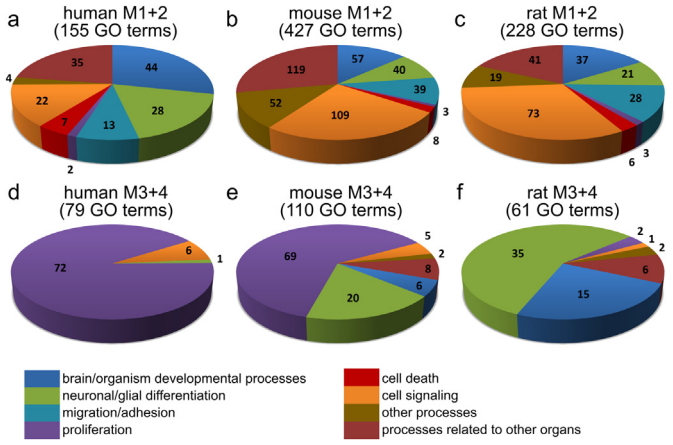

Fig. 3. Classification of GO terms into superordinate biological processes. ORA analysis was performed using the Cytoscape plugin ClueGO (Bindea et al., 2009). All overrepresented GO terms ($p \leq .01$ based on a two-sided hypergeometric option with a Bonferroni correction) for modules 1 + 2 (a-c) and modules 3 + 4 (d-f; also see Fig. 2) were summarized into 8 superordinate processes for human (a, d), mouse (b, e) and rat (c, f) NPC based on expert judgment. Number in the pie chart represents the number of GO terms assigned to each superordinate processes.

functional "Neurosphere Assay". Here, the processes NPC proliferation, migration, neuronal and glial differentiation as well as cell death are represented by regulation of GO terms such as cell proliferation (GO:0042127), cell motility (GO:0048870), neurogenesis (GO:0022008), axonogenesis (GO:0007409), gliogenesis (GO:0042063) or regulation of programmed cell death (GO:0043067; comprehensively shown in Table A. 4). To visualize how GO term enrichment compares in-between the three species, we summarized all GO terms into the following eight superordinate processes by expert judgment similar to Waldmann et al. (2014): (i) brain/organism developmental processes, (ii) neuronal/glial differentiation, (iii) migration/adhesion, (iv) proliferation, (v) cell death, (vi) cell signaling, (vi) other processes and (vii) processes related to other organs (Table A. 5–7). Fig. 3a-c demonstrates that from the temporal expression cluster M1 and M2, GO terms associated to 'brain/organism developmental processes', "neuronal/glial differentiation" are the most represented and together with "migration/adhesion", 'proliferation and cell death' make up 60% of all GO terms in hNPC, while these GO terms represent only 34% and 41% in mNPC and rNPC, respectively. GO terms associated with processes related to cell signaling correspond to 14% of all GO terms in hNPC, while this is the largest group in rodent NPC with 26% (mNPC) and 32% (rNPC) of all GO terms. In total numbers, only 31 GO terms are associated to this group in hNPC, 92 in rNPC and 164 in mNPC. Some of the processes present in rodent but not in hNPC are e.g. cell-cell signaling (GO:0007267), response to steroid hormone (GO:0048545), negative regulation of cell communication (GO:0010648) or protein phosphorylation (GO:0006468; Table A. 4). The remaining 26% (hNPC), 40% (mNPC) and 26% (rNPC) GO terms are associated to other processes (e.g. single-organism biosynthetic process, GO: GO:0044711 or ion transport, GO:0006811) and processes in other organs. One example for the latter group is the process heart development (GO: 0007507). Although this is prima vista not related to brain development, it shares 41 of the 62 genes (66%) with the process nervous system development (GO:0007399) in hNPC (Table A. 1). This example demonstrates that GO terms of processes related to other organs might be overrepresented due to shared genes with nervous system development.

Next, we computationally analysed the biological functions of genes in clusters M3 and M4. From the 404 DEX genes in hNPC, 906 in mNPC and 1417 in rNPC, respective 345 (85.4%), 761 (84.0%) and 1058 (74.7%) were annotated to 79 (hNPC), 110 (mNPC) and 61 (rNPC) GO terms with 42% shared GO terms between hNPC and mNPC and none between hNPC and rNPC (Table 1; Table A. 8, 10–11). There are two- (mNPC) to three-fold (rNPC) more DEX genes present in rodent than in hNPC pointing to differences in culture maturation and/or species differences. This is supported by the magnitude fold change in DEX genes in rodents, mainly rat NPC, which display higher mean gene expression regulations compared to hNPC (mean regulation of M3: 3.5 fc in hNPC, 4.7 fc in mNPC, 7.1 fc in rNPC; Fig. 2h). Fig. 3d-f demonstrates that almost all GO terms in hNPC (91%) and more than half in mNPC (69%) are associated to the process of cell proliferation such as cell cycle (GO:0007049), chromosome organization (GO:0051276) or mitotic nuclear division (GO:0007067). Within these GO terms 60–100% of all associated genes are down-regulated (Table A. 8 + 10). This observation suggests that cell proliferation is still an important process during the onset of differentiation but strongly decreases between day 3 and 5 in hNPC and mNPC. Other GO terms in mNPC (18%) and more than half in rNPC (57%) are associated with the progression of cell differentiation and maturation. Here some GO clusters are cilium organization (GO:0044782) in mNPC, determination of left/right symmetry (GO:0007368) or microtubule-based process (GO:0007017) in both species and cilium morphogenesis (GO:0060271) or centriole assembly (GO:0098534) in rNPC (comprehensively shown in Table A. 10–15). Because the majority of DEX genes in M3 and M4 of hNPC were dominated by downregulated genes, we performed an ORA for upregulated genes (M3) separately. ORA of hNPC M3 shows that 16 processes involved in maturation such as axonogenesis (GO:0007409) and dendrite development (GO:0016358) are overrepresented (Table A. 9) in our data set.

Overall, the ORA of GO BP especially from M1 and M2 reflects (i) the multicellularity of the 3D neurospheres and (ii) specific neurodevelopmental processes during NPC development. These results demonstrate that molecular signatures of gene expression changes line the functional processes that are studied in the frame of the "Neurosphere Assay" in vitro over NPC differentiation. It also indicates that many of these major processes of brain development and cell organization are conserved across species, yet with distinct molecular signatures. However, there are considerable species differences in the abundance of processes related to cell signaling, proliferation or the progression of cell maturation between the in vitro systems of the three species.

3.3. Identification of key regulators for human neurodevelopmental processes

To identify the underlying genes and pathways of the neurodevelopmental processes studied with the "Neurosphere Assay", we performed a clustering based on shared genes between GO terms overrepresented in modules M1 and M2 of hNPC (Fig. 4) and rodent NPC (Fig. B. 2–3). From the 19 GO clusters in hNPC we extracted those representing the major neurodevelopmental processes, i.e. cell migration, neurogenesis and gliogenesis. As the cluster neurogenesis was included in a cluster with general GO terms on (neuro)development, we

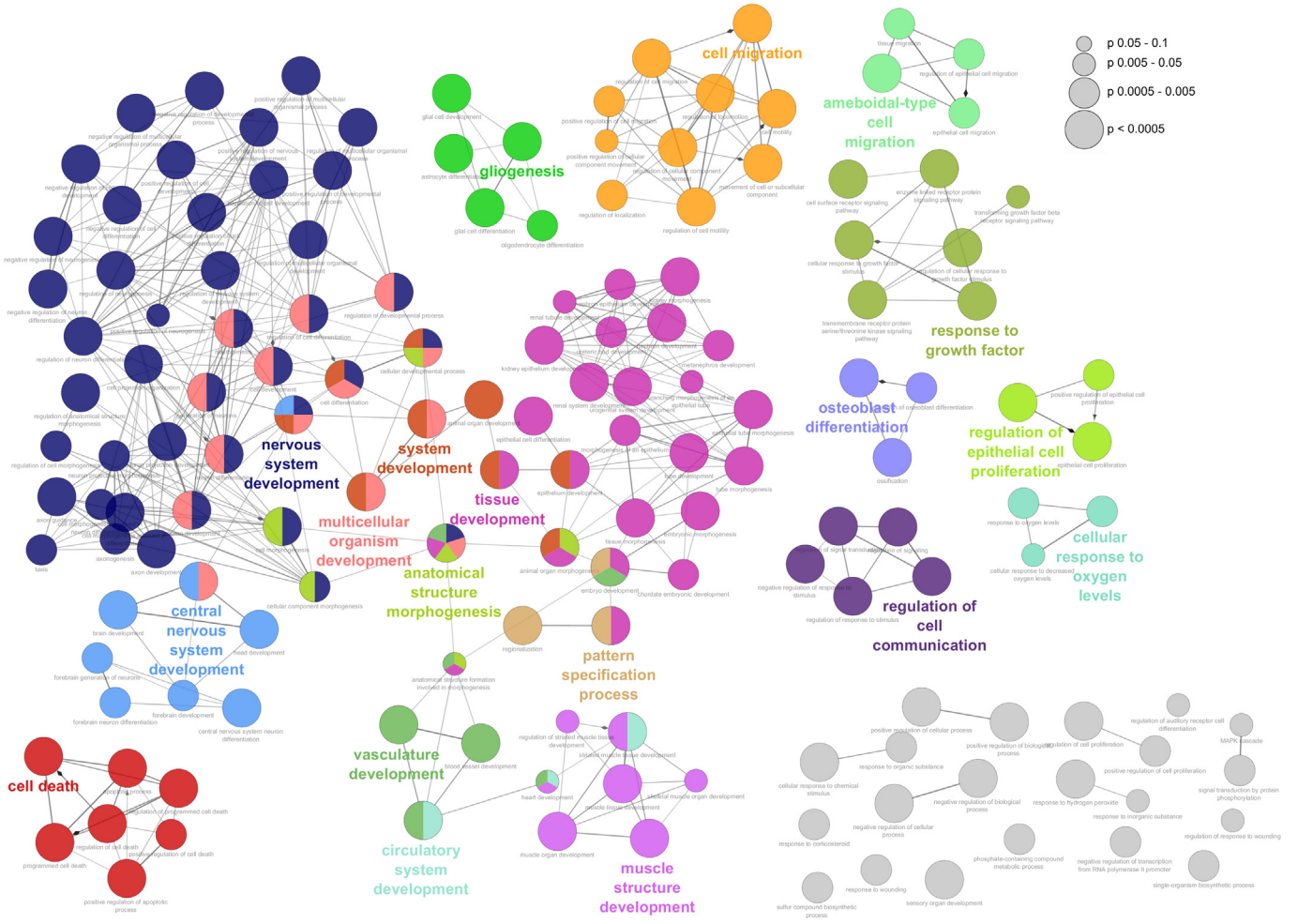

Fig. 4. GO clustering in hNPC.
Overrepresented GO terms of modules 1 and 2 in hNPC were clustered according to gene overlap between GO terms with a kappa score threshold of 0.5 and at least three GO terms within one cluster using the Cytoscape plugin ClueGO (Bindea et al., 2009). Edge thickness represents similarity between GO terms. Node size represents significance of overrepresentation. The GO term with the highest significance determines the name of the respective GO cluster (bold; colored). Different colors represents different GO cluster. Grey nodes do not belong to a cluster. Significance thresholds of ORA was set to $p \leq .01$ based on a two-sided hypergeometric option with a Bonferroni correction.

manually removed all GO terms, e.g. GO terms like brain development (GO:0007420), regulation of cell morphogenesis (GO:0022604) or cell development (GO:0048468) that were not directly related to neurogenesis (Fig. B. 4).

All genes within each cluster were enriched for their interaction (binding, activation, expression and inhibition) based on information from STRING protein-protein Interaction database (Szklarczyk et al., 2015); http://string-db.org; Fig. 5. We identified highly connected genes as key regulators (KR) or hubs for individual human neurodevelopmental processes. Genes that show at least three times (migration, neurogenesis) or two times (gliogenesis) the number of connections compared to the mean number of connections per gene within a cluster were defined to be highly connected. Thereby, we identified *BMP2*, *EGFR*, *MYC* and *NOTCH1* as KR across all three processes, *VEGFA*, *JUN* and *FGFR1* as KR for migration and neuronal differentiation and *EPHA2*, *LYN*, *PDGFRB*, *SRC* only for migration (Fig. 5; Table 2). Species comparison revealed that for mNPC and rNPC only few genes (between 7% to 14%) of the processes migration, neurogenesis and gliogenesis with interaction data are shared with hNPC. From the KR only *VEGFA* is present in the two species, a finding that could be explained by the general low number of DEX genes shared between the species. The KR for rodent NPCs are *Agt*, *Cav*, *Flt*, *Fyn*, *Itga*, *Pdgfb*, *Ptgs2* for migration in mNPC, *Cx3cr1*, *Flt1*, *Itgb4* and *Ptk2* for migration in rNPC, *Bmp4* for neurogenesis in mNPC and *Ptk2* for neurogenesis in rNPC. For the process gliogenesis there was no KR for mNPC and the processes did not appear as a separate cluster in rNPC (Table A. 20–24; Table 2).

For functional validation of some of the KR identified in hNPC transcriptomes, we analysed the effects of their pharmacological modulation on migration, neuronal and oligodendrocyte differentiation in all three species. As KR we chose *BMP2*, *EGFR* and *NOTCH*, as they were predicted to modulate all three processes, i.e. hNPC migration, neuronal and glia differentiation (Fig. 5; Table 2). We modulated BMP2 signaling by addition of 0.01–100 ng/mL BMP2 during differentiation of human, mouse and rat neurospheres (Fig. 6). BMP2 did not affect migration of human and mNPCs, but induced migration of rNPCs after 72 h (149.9 ± 7.2% of control at 10 ng/mL; Fig. 6a). Furthermore, BMP2 did not affect differentiation into βIII-tubulin positive neurons in hNPC, while it induced and reduced neuronal differentiation in mouse and rNPC, respectively (202.1 ± 46.9% of control at 50 ng/mL in mNPC and 62.2 ± 8.6% of control at 5 ng/mL in rNPC; Fig. 6b). BMP2 reduced the differentiation to O4 positive cells in all species (48.7 ± 1.8% and 50.8 ± 3.5% of control at 1 ng/mL in human and rNPC, respectively and 54.2 ± 4.4% of control at 5 ng/mL in mNPC; Fig. 6c). In addition, BMP2 induced maturation of GFAP positive cells, as indicated by a concentration-dependent increase of mature astrocytes at the expense of radial glia cell in the migration are. This effect

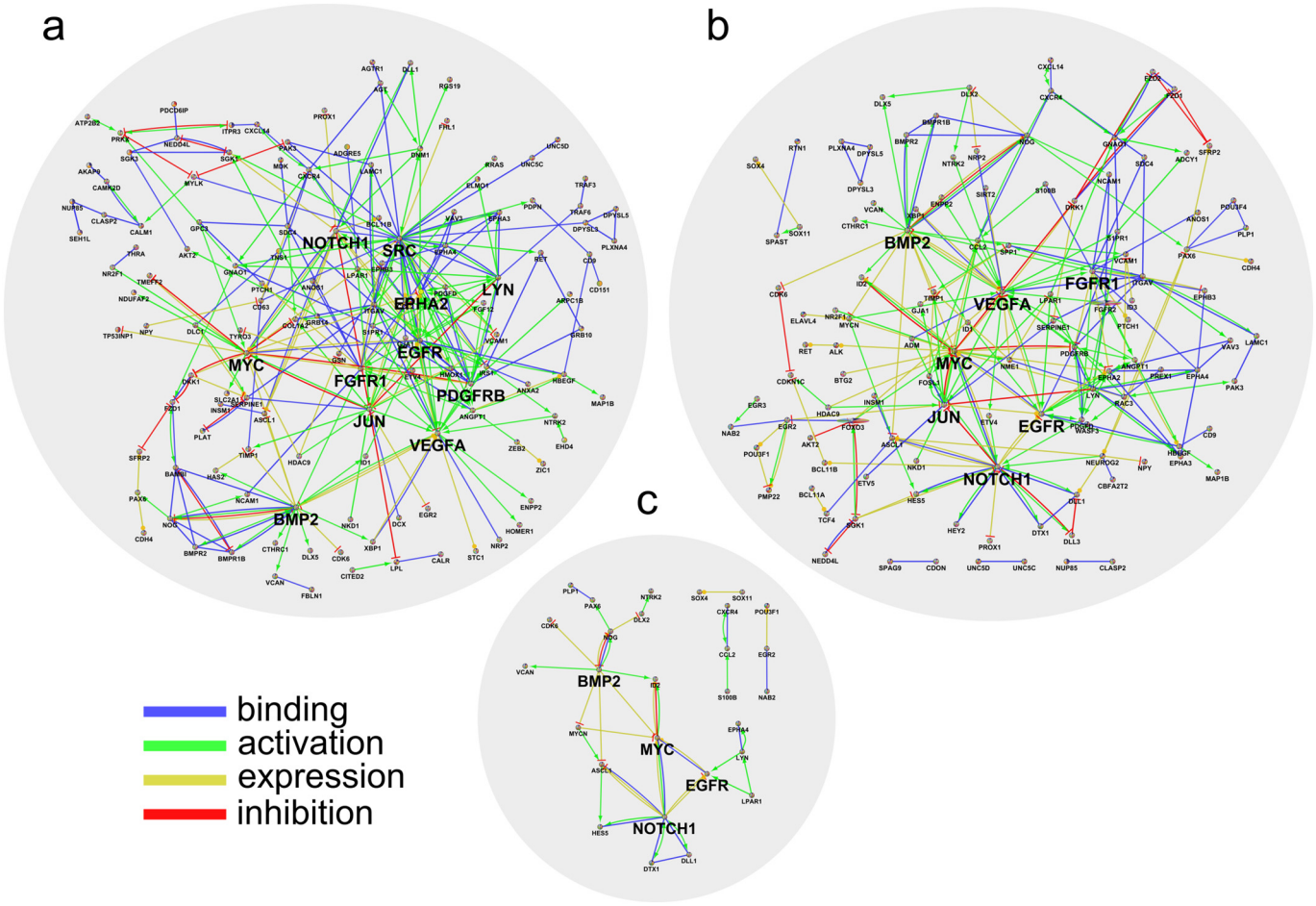

Fig. 5. Gene-gene interaction networks of major neurodevelopmental processes overrepresented in hNPC.
The cytoscape plugin GluePedia (Bindea et al., 2013) was used to enrich the GO clusters cell migration (a), neurogenesis (b) and gliogenesis (c) from the ORA of modules 1 and 2 of hNPC with information on gene-gene/protein-protein interactions (binding in blue, activation in green, expression in yellow and inhibition in red) from the STRING database (Szklarczyk et al., 2015) with a confidence score of ≥0.75. Highly connected genes (bold) were identified as those genes showing at least three times (migration, neurogenesis) or two times (gliogenesis) the number of connections compared to the mean number of connections per gene within the respective cluster. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was quantified by measuring the reduction of radial glia cell migration in comparison to the total migration of GFAP positive cells (from 82 ± 6.2% of total migration at control to 62.4 ± 0.9% of total migration at 1 ng/mL; Fig. 6j + k). Because migrating rodent NPC did not display typical radial glia morphology in our neurosphere culture (Baumann et al., 2015), BMP2 effects on astrocyte maturation was only studied in hNPC. All effects described for BMP2 were at concentrations that did not affect overall cell viability measured by mitochondrial activity (Fig. B. 5a + c).

NOTCH signaling was modulated by the addition of 0.08–5 μM of the NOTCH inhibitor DAPT. NOTCH inhibition did not affect migration of human and rNPC, but inhibited migration of mNPC after 72 h (76 ± 5.7% of control at 5 μM; Fig. 6d). Differentiation into βIII-tubulin positive cells after 72 h was not affect by NOTCH inhibition (data

Table 2
Highly connected genes as key regulator for neurodevelopmental processes.

		Migration	Neurogenesis	Gliogenesis
Human	MEAN #connections/Gene Human	4.3	5.7	3.4
	Key regulators (#connections) human	BMP2*(23), EGFR*(22), EPHA2(16), FGFR1(18), JUN (20), LYN(14), MYC*(23), NOTCH1*(18), PDGFRB(18), SRC(44), VEGFA(26)	BMP2*(24) EGFR*(17), FGFR1(14), JUN (20), MYC*(25), NOTCH1*(26), VEGFA(26)	BMP2*(10), EGFR*(6), MYC*(10), NOTCH1*(14)
Mouse	MEAN #connections /Gene (overlap human in %)	4.4 (13.6%)	2.7 (14.4%)	3.3 (7.1%)
	Key regulators (#connections) mouse	Agt(17), Cav1(20), Flt1(14), Fyn(17), Itga(17), Pdgfb (14), Ptgs2(15), Vegfa(17)	Bmp4(10)	none
Rat	MEAN #connections/Gene (overlap human in %)	2.7 (8.2%)	2.5 (7.6%)	not present
	Key regulators (#connections) rat	Cx3cr1(10), Flt1(11), Itgb4(9), Ptk2(16)	Ptk2(9)	

* Highly connected in all three neurodevelopmental processes.

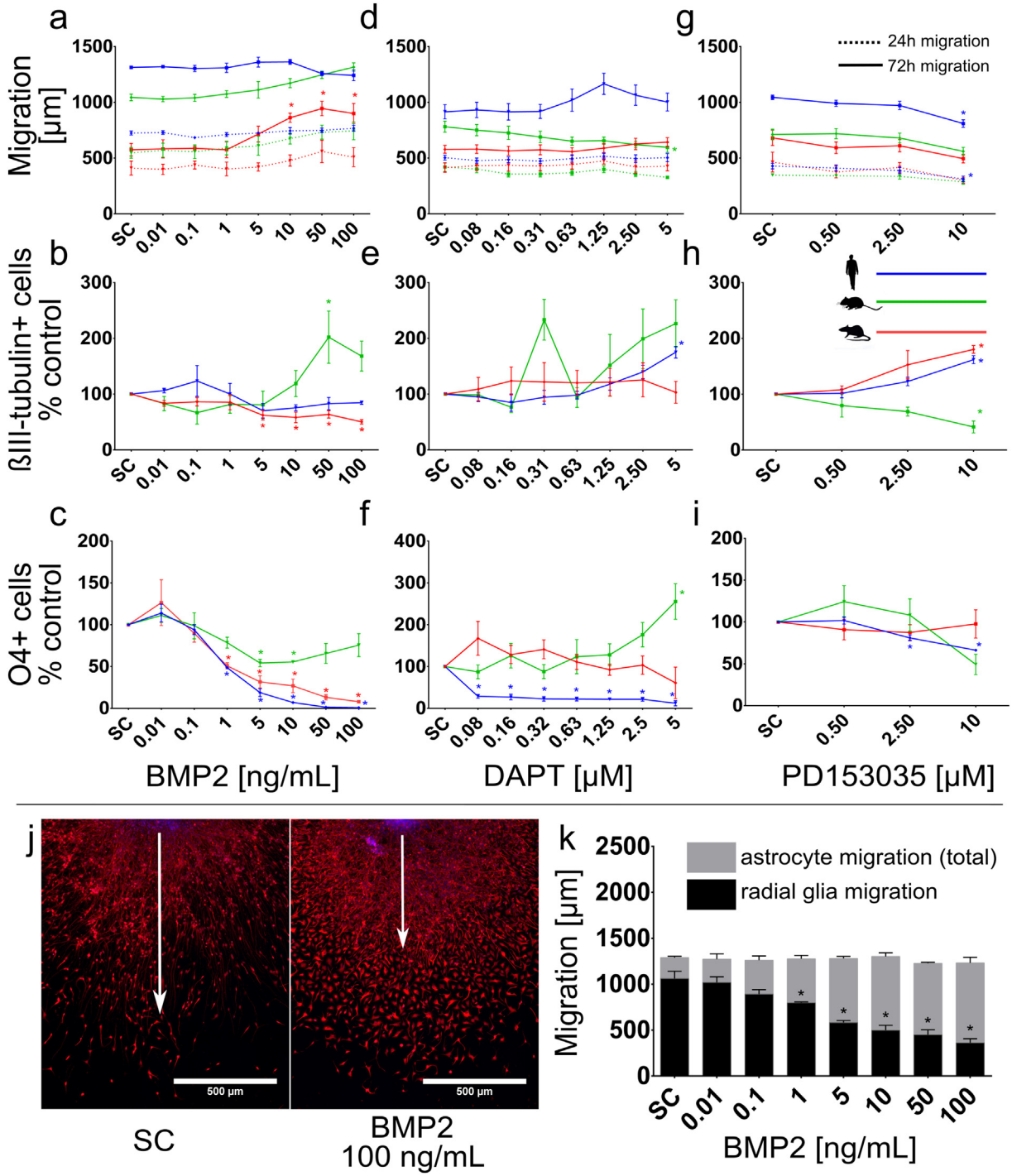

Fig. 6. Pharmacological modulation of the BMP2, NOTCH and EGF pathway.
NPC from human (blue), mouse (green; differentiated in FCS) and rat (red) were treated with increasing concentrations of BMP2 (a, b, c), DAPT (NOTCH inhibitor; d, e, f) and PD1530353 (EGFR inhibitor; g, h, i) and analysed for migration (after 24 h (dotted line) and 72 h (solid line); a, d, g) neuronal differentiation (after 72 h for BMP2, EGFRi and 120 h for DAPT; b, e, h), oligodendrocyte differentiation (after 120 h; c, f, i) and astrocyte maturation (only in hNPC after BMP2 treatment; j, k). Neurons and oligodendrocytes were immunocytochemically stained with ßIII-tubulin and O4, respectively, and quantified as percent of neurons/oligodendrocytes compared to Hoechst33258 counterstained nuclei. Astrocytes were stained with GFAP and maturation of radial glia cells was measured as migration of radial glia compared to total migration of GFAP positive cells. (j) Representative pictures of immunocytochemically stained astrocytes after 72 h BMP2 treatment of hNPC. Scale bar represent 500 µm, white arrow marks radial glial migration. Except for migration distance (shown as raw migration distance in µm), data was normalized to the solvent control and is displayed as concentration response relationship with mean ± SEM of at least three independent experiments. * indicates a significant difference to solvent control based on one-way ANOVA ($p < .05$) followed by Dunnett's multiple comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

not shown). NOTCH inhibition induced neuronal differentiation only of human, not rodent NPC after 120 h (175.2 ± 10.5% of control at 5 µM, Fig. 6e), while after 72 h DAPT exerted no effects on neuronal differentiation of either species. Differentiation of hNPC into O4 positive oligodendrocytes was inhibited by NOTCH inhibition (28.5 ± 4.3% of control at 0.16 µM DAPT), not affected in rNPC and

induced to 255.4 ± 42.6% of control in mNPC (5 μM DAPT, Fig. 6f). However, these additionally formed mouse O4 positive cells did undergo apoptosis as identified in Fig. B. 5 k + l, leaving non-apoptotic oligodendrocytes at the same number than the control cultures. In differentiated human or rat NPC, no apoptotic O4 positive cells were present. The highest concentrations of DAPT (1.25–5 μM) reduced mitochondrial activity in all species (Fig. B. 5e). Except for rNPC at 5 μM DAPT, this effect was not accompanied by a reduction in number of nuclei (Fig. B. 5f), which lead us to the assumption that DAPT reduces mitochondrial activity rather than affect cell viability.

Intrinsic EGF signaling was inhibited by addition of 0.5–10 μM of the EGFR inhibitor PD1530353. Inhibition of EGFR reduced migration of hNPCs after 24 h and 72 h (75.0 ± 5.9 and 77.5 ± 3.2% of control at 10 μM) and did not affect migration in m- and rNPC (Fig. 6g). Differentiation to βIII-tubulin positive cells was induced in h- and rNPC (161.9 ± 7.2% and 180.2 ± 7.0% of control at 10 μM) and reduced in mNPC (41.4 ± 10.8% of control at 10 μM; Fig. 6h). PD1530353 reduced the formation of O4 positive cells in hNPC (66.3 ± 1.4% of controls at 10 μM), while it did not affect the differentiation to O4 positive cells in m- and rNPC (Fig. 6i). Effects on migration in hNPC and neuronal differentiation in all species was accompanied by a reduction in number of nuclei (59 ± 9.0%, 72 ± 5.3% and 78 ± 6.0% of control in h-, m- and rNPC, respectively). In rNPC the 10 μM PD1530353 additionally affected viability after 72 h (75.6 ± 9.0% of control).

4. Discussion

Within the paradigm shift of 'Toxicology in the 21st century' the need for in vitro assays is voiced that reliably predict human toxicity (Collins et al., 2008; NRC, 2007). One of the required toxicity endpoints with regard to chemical safety is reproductive and developmental toxicity currently assessed with the extended one-generation study (OECD, 2012). While giving valuable information for many different endpoints, this bioassay as well as the OECD TG426 specifically designed for DNT evaluation are not sufficient for identifying neurodevelopmental toxins (Fritsche et al., 2017; Tsuji and Crofton, 2012). Therefore, alternative assays predicting (neuro)developmental toxicity also for regulatory applications are urgently needed (Bal-Price et al., 2015; Fritsche et al., 2017; Bal-Price et al., 2018). For any application, a thorough understanding of the alternative test system especially on the molecular level increases confidence in the method and might allow future usage in a broader context like the 'integrated approaches for testing and assessment' (IATA; Judson et al., 2013; National Academy of Sciences, 2007; Roggen, 2011). This is why in this work the molecular equipment (transcriptome) of developing human NPC over time (proliferating versus 3 and 5 days differentiated cells) was assessed; they were compared to time-matched (www.translatingtime.org; Workman et al., 2013) mouse and rat NPC transcriptomes to identify species-specificities. Pathways recognized as major regulating hubs were functionally validated for their impact on NPC migration, neuronal and oligodendrocyte differentiation across the three species (Fig. 1).

Recently it was recognized that the uniqueness of higher cognitive and emotional functions in humans is largely determined by human-specific neurodevelopmental gene expression (Dehay and Kennedy, 2009). In addition, transcriptome analyses of developing brain cells from mouse embryonic stem cells (mESC) were shown to recapitulate early neurodevelopmental stages in vitro and were thus identified as reference points for DNT testing (Abranches et al., 2009; Kuegler et al., 2010; Zimmer et al., 2011). This concept was transferred to their human correlates (hESC; van de Leemput et al., 2014) and such hESC were applied successfully in a transcriptome-based compound evaluation for the two developmental neurotoxicants methylmercury and valproic acid (Krug et al., 2013). Moreover, transcriptome analyses of neural differentiating human induced pluripotent stem cells are applied for disease modelling (Boland et al., 2017) strengthening human relevance of such an approach.

On this basis, we studied gene expression in neurodevelopmental in vitro systems of three different species. During NPC differentiation in vitro, 1684 human, 1979 mouse and 2324 rat genes were DEX (> 2-fold up- or downregulated; $p < .01$; Fig. 2a-c). In a study that analysed transcriptional changes of mouse differentiating NPC after treatment with brain derived neurotrophic factor (BDNF) or neurotrophin 4 (NT4) a total of 722 and 624 genes were differentially expressed at any of the three time points (24 h, 48 h or 96 h; van Dartel et al., 2010). The lower number of genes can be explained by the use of different microarray chips with less transcripts (13627) compared to the chips used in this study (> 20,000). Other similar studies were performed analyzing transcriptome changes of human embryonic stem cells (ESC) differentiating to cardiomyocytes (CM; Meganathan et al., 2012). Between undifferentiated hESC and 48 h or 12 days differentiation towards the CM lineage, respective 3579 (van Dartel et al., 2010) and 3035 (Meganathan et al., 2012) transcripts were found to be differentially expressed. These are around twice as many gene changes as we found in undifferentiated compared to 3 or 5 days differentiated hNPC (Fig. 2a-c). This might be because NPC are already on their way to neural tissue, while hESC are still omnipotent and thus differ more strongly from the terminally differentiated cells.

Principle Component Analyses using all genes that are significantly changed on the arrays ($p < .01$) and present in all three species (5570) revealed that the majority (56.7%) of all variance between the condition and species can be described by the first two principal components (Fig. 2f). The PCA plot clearly shows the differentiation dynamics of the NPC in vitro system and indicates that the variance within experimental groups is relatively small compared to the variance between time points and species (Fig. 2f). A similar PCA pattern was observed during hESC differentiation to CM. Here, 24 and 48 h (van Dartel et al., 2010) as well as 6 and 20 days of differentiation (Li et al., 2015) were clearly distinguishable from the stem cells of origin also pointing to highly dynamic in vitro systems with regards to differentiation capacities. Besides differences in gene expression over time within one species, we also observed well-defined distinctions in gene expression differentiation dynamics between the three species (Fig. 2f). Comparison of human and mouse ESC differentiated to embryonic bodies revealed that out of a total of 903 GO terms (biological processes), gene expression was only correlated between species in 395 and not correlated in 508 GO terms indicating substantial differences in transcriptional regulation of ESC-based embryonic body formation (Sun et al., 2007). This work supports the observed species differences in gene expression profiles during NPC development presented here.

One striking aspect of this study is that only approximately 10% of all DEX genes (> 2-fold, $p < .01$; Fig. 2c) over developmental time were common in all three species (Fig. 2e). This is a very small number considering that these in vitro systems are functionally very similar, i.e. migrating and differentiating primary NPC (Baumann et al., 2015). These 186 genes cluster in 61 GO Terms (Table A. 18) that contain some specific neurodevelopmental processes, but also a large variety of non-neurodevelopmental-related biological functions. A large variety of genes in these non-specific GO Terms, however, are generally involved in tissue and organ development pointing to the fundamental biological significance of these molecules in developing cell functions. Such include MYC (present in 18 of these GO Terms), which is engaged in cellular signaling including cell proliferation (Hydbring et al., 2017), PDGFRB (present in 16 of these GO Terms), which guides a variety of developmentally-relevant signaling pathways (Demoulin and Essaghir, 2014) and FGFR2 (present in 31 of these GO Terms), that obtains multiple functions during organ development (Goetz and Mohammadi, 2013) including the brain. A comprehensive list of the commonly regulated genes and their grouping into GO Terms for biological processes can be found in Table A 18.

The DEX genes that differ between species, however, enrich in GO Terms for biological processes that were assembled into analogous

superordinate processes by expert judgment (Fig. 3). These GO terms in general reflect the neurodevelopmental processes that we study with the "Neurosphere Assay" on a functional level in vitro (Baumann et al., 2015; Moors et al., 2009) and were previously identified in the transcriptomes from developing brains in vivo (Matsuki et al., 2005). Despite the similar grouping of enriched GO terms in superordinate processes the question remains why the majority of DEX genes diverge between the species. There are three major reasons that might explain why DEX genes of human, mouse and rat NPC differ considerably over time despite qualitative similarities in GO Term clusters. The first explanation might lie in species differences in developmental timing, the second one in differences in molecular equipment and/or regulation of equivalent cells and the third one in different cell type compositions of brains from different species. It is highly likely that the results presented in this study are motivated by a mixture of all three arguments and we will now provide examples for each of them.

Timing of brain development is known to follow different temporal traits in diverse species (Rice and Barone, 2000; Semple et al., 2013; Workman et al., 2013). During human brain development, changes in the transcriptome are largest during the fetal period, i.e. 9 out of 10 genes are DEX between different developmental time points in vivo (samples taken every 2–5 post conceptual weeks (PCW) starting from PCW 4) and/or brain regions demonstrating high gene expression dynamicity during pre- and early postnatal development (Kang et al., 2011). Rodent brain development underlies similar gene expression changes than ontogenesis of the human organ, yet at a more rapid pace (Chou et al., 2016; Goggolidou et al., 2013; Jaffe et al., 2014). In addition, neurodevelopmental processes that are guided by gene expression changes take place at species-specific speeds as exemplified by oligodendrogenesis, which takes 5 days in rodents, but 11–12 weeks in humans (Barateiro and Fernandes, 2014) leading to differences in oligodendrocyte-related gene expression between species also during NPC development in vitro when analyses are performed at the same time points (Baumann et al., 2015; Dach et al., 2017). Species- and cell type-specific developmental speed might also explain that human, and not rat NPC down-regulate proliferation-related genes between 3 and 5 DIV. Here, more experimental work with a higher time resolution is needed that scrutinizes proliferation of NPC and developing astrocytes in the in vitro systems. In the mouse, the situation might be different due to cell culture in FCS-containing medium as FCS strongly influences neural cells' proliferation and differentiation behavior in a concentration-dependent manner (Budhram-Mahadeo et al., 1994). FCS concentrations used in this study (1%) did not inhibit NPC differentiation. Taken all timing aspects together, it might not be surprising that gene expression profiles from identical cell types like here the primary NPC from different species gained from brains at corresponding time points (Workman et al., 2013; Fig. 1) display distinct transcriptomes in their undifferentiated state as well as during in vitro differentiation over time (Fig. 2f).

The second aspect underlying the observed species differences might be differences in molecular equipment and/or regulation of genes within cell types. A recent study revealed substantial cross-species differences between humans and mice with a dramatic shift of cortical layer-specific gene expression patterns between species indicating cross-species conservation and divergence of gene expression at anatomical and cell type levels (Zeng et al., 2012). One example from this study is *CALB2*, which is preferentially expressed in the ventricular zone/sub ventricular zone-originated interneurons and was found enriched in human compared to mouse brains (Zeng et al., 2012). Direct comparison of expression between species cannot be solely assessed by DEX genes, which describes expression changes between two time points and is a known issue when dealing with cross-species transcriptome comparisons (Lu et al., 2009). Therefore, we defined a gene as likely to be expressed (present) or likely to be not expressed (absent) based on criteria adapted from Kang et al. (2011; median log2 intensity value in 0-day samples is ≥ 6 = present, otherwise absent). We are aware of the technical limitations of absolute thresholds when using Affymetrix arrays intensity information, which is why the following comparisons can solely provide an indication that needs further experimental proof. With these combined methods we found that *CALB2* expression and regulation over time seems to be human NPC-specific in our data set (Fig. 2g and Table A. 19). In addition, *PDGFD* acting through *PDGFRB* regulates cell-cycle progression and progenitor cell expansion in human, but not mouse, cortex (Lui et al., 2014). This instance is also reflected in our species-overarching in vitro methods with PDGFD seeming to reach the "present" threshold in hNPC, but not in mouse correlates (Fig. 2g and Table A. 19). Surprisingly we observed rNPC to be similar to hNPC with regard to this marker, but to the best of our knowledge, there is no literature available to compare our findings with. Concerning genes expressed during neuro-, astroglio- and oligodendrogenesis, we also found similarities and differences in gene abundance across species. The human astrocyte-specific gene LRRC3B, for example, seems to be exclusively present and regulated in human compared to rodent cultures, while the astrocyte maturation markers *SPARCL1*, *GFAP* and *S100b* seem to be present in NPC from all three species (Zhang et al., 2016); Fig. 2g and Table A. 19. These examples support our second notion that some of the transcriptome differences that we observe between human, mouse and rat neurospheres might be due to qualitative species-specificities.

The third explanation for the large species differences in DEX genes identified in our study might root in a different cell type composition of human compared to rodent brains. One striking macroscopic distinction of human from rodent brains is the brain surface. While human brains are gyrencephalic, rodent brains contain no gyri and sulci and are thus lissencephalic. Gyrencephaly is a result of cellular expansion of a special type of progenitor cell, the basal progenitor (BP) or outer radial glia (oRG) cell in the human enlarged outer subventricular zone (OSVZ) of the developing cortex (Borrell and Götz, 2014; Lui et al., 2011; Pollen et al., 2015; Silbereis et al., 2016; Thomsen et al., 2015). This cell type is thought to be specific for human brains and can thus be identified by human-specific molecular markers. One of these markers is the recently identified oRG cell marker *FAM107A* (synonym for DRR1; Lui et al., 2014; Pollen et al., 2015; Thomsen et al., 2015), which we found to be expressed in human and absent in mouse NPC, supporting the previously published data. However, we also found this marker to be present and highly regulated in rat NPC (Fig. 2g and Table A. 19). One reason for the absence of expression and regulation of *FAM107A* in developing mouse and the expression and regulation in developing rat NPC could be the neuronal expression in mouse E10.5–16.5 and rat E18.5 brains (Asano et al., 2010). While neuronal *fam107a* expression is restricted to an early embryonic timeframe and the NPC used in this study are generated from PND1 brains, mNPC do not show any expression of this gene. In rat, neuronal *fam107a* expression starts on E18.5 in vivo suggesting that our observed gene presence in developing rat NPC is due to neuronal and not radial glia cell expression, which needs further experimental confirmation. These data indicate, that a combination of developmental timing and cell type-specific expression might drive gene abundance in such mixed cell type systems.

In contrast to the oRG, ventricular zone RG (vRG) are supposedly more similar across species (Dehay and Kennedy, 2007). This seems to be reflected on the molecular level within this study as the vRG-specific *CRYAB* and *ANXA1* gene products (Thomsen et al., 2015) seem to be present in NPC of all three species (Fig. 2g and Table A. 19). Intermediate progenitor cells (IPC), however, likely seem to beabsent in the culture due to absence of the IPC marker *EOMES* (TBR2), despite *HES6* presence in mouse and human cells (Thomsen et al., 2015; Fig. 2g). *HES6*, however, seems to have additional, *NFκB*-dependent functions in migrating neurons during cortical development (Methot et al., 2013) suggesting that *HES6* expression in those neurospheres is likely due to expression in migrating neurons and thus independent of IPC. In addition, all NPC express *HES1*, *VIM*, *SLC1A3* (*GLAST*) and *NES* (Borrell and Reillo, 2012; Lui et al., 2011; Pollen et al., 2015) supporting the

concept that also a large variety of genes are expressed in a similar fashion across species (Zeng et al., 2012). However, the here discussed data also provides evidence that some of the transcriptional species differences observed in this study are based on different cell types, i.e. oRG cells in humans (Pollen et al., 2015; Thomsen et al., 2015), which seem to be well-reflected in the neurospheres in vitro systems.

Besides the GO term grouping by expert judgment, we analysed transcriptomes from each species across differentiation time using a GO term clustering in the Cytoscape plugin ClueGO (Bindea et al., 2009; Fig. 4). The plots – and here especially the human data - illustrate that the transcriptomes mirror the in vitro functions of the 3D models, i.e. NPC proliferation, migration, differentiation into neurons and glia cells as well as apoptosis over 5 days of differentiation (Fig. 4, Fig. B 2 + 3). Comparison of the plots of the three species reveals differences in their appearance (Fig. 4, Fig. B. 2 + 3). This is probably due to two reasons. For one, attributable to the approximate 90% differences of DEX genes between species (Fig. 2C), distinct GO terms were annotated from the data sets. Secondly, existing GO term annotations for humans, mice and rats differ in quality and quantity due to the different background information available for each species (Rhee et al., 2008). As an example, the biological process “cell signaling” is defined by four times more GO terms in mouse than in human NPC-derived transcriptomes (Fig. 2). This is probably owing to the large amount of existing signaling data generated in mice compared to humans including data from the many transgenic mice that dominate biomedical research (Ormandy et al., 2009). Despite several limitations, this kind of GO analysis is still useful to point out major differences in regulated genes and the processes behind them as done in this manuscript.

For a pathway-to-function validation and species comparison we picked the three key regulators *BMP2*, *EGFR* and *NOTCH* that were computationally predicted to be involved in NPC migration, neuro- and gliogenesis in hNPC (Table 2). The data of the consequences of *BMP2*, *NOTCH* and *EGFR* pathway modulation are shown in Fig. 6.

The transforming growth factor-β (TGF-β) superfamily member BMP2 signals through BMP receptors (BMPR) Type 1 (BMPR1a/Alk3 and BMPR1b/Alk6) or Type 2 (BMPR2; Bond et al., 2012). According to the human and rodent transcriptomes, with the exception of *bmpr1b* in rat NPC, these *BMPR* are present in proliferating and differentiating cultures (Table A. 19). In contrast to the in silico prediction (Fig. 5a-c), BMP2 only acts on glia-related endpoints in human NPC by reducing oligodendrocyte differentiation (Fig. 6c) and accelerating astroglial maturation (Fig. 6j + k). In addition to reduction of oligodendrocytes, BMP2 induces migration and reduces neuronal differentiation of rat and induces neurogenesis in mouse NPC (Fig. 6a-c), yet BMP2 was not predicted as a modulator of any of these endpoints in the rodent cultures (Table A. 21–24). Discrepancies of computational prediction based on GO terms and actual experimental data when using relevant cell systems might be built on the data behind GO annotations. Such data underlying GO terms are retrieved from different tissues, cell models, in vitro and in vivo analyses, species, and, in case of development, distinct developmental timing (Rhee et al., 2008). For example, in our data set gene expression of *ID1* (*inhibitor of DNA binding/differentiation*) strongly increases during differentiation in hNPC (9-fold at 0vs3 days). Because BMP2 is a known transcriptional inducer of *ID1* (Katagiri et al., 2002) and both of them are annotated to the GO term cell migration (Table A. 1), this annotation contributed to the computational identification of BMP2 as a hub gene for cell migration. Searching for the data behind this annotation, the information on *ID1* and migration is a ‘traceable author statement’ based on observations in endothelial cells (Goumans et al., 2003) and is not related to migration in the developing brain. The information that *BMP2* is related to migration is inferred from sequence or structural similarity of homolog or ortholog genes, which is a hypothesis that has no experimental proof. Consequently, data sets behind GO terms need improvement with regards to specificity of cells, tissues, species and, if applicable, developmental timing. Nevertheless, we demonstrated that BMP2 effects on rodent NPC are similar than previously published and showed the new information that BMP2 induces rat NPC migration. BMP2 induces E13.5 (mouse) and E16 (rat) NPC differentiation into βIIItubulin+ neurons (Liu et al., 2013; Mabie et al., 1997; Mehler et al., 2000) or reduces this process in mouse E17 NPC (Gross et al., 1996) and mouse embryonic stem cells (Gossrau et al., 2007), yet in all systems BMP2 impedes oligodendrogenesis and in all but the mouse E13.5 BMP2 promotes astroglial fate (summarized in Table A. 25). Thus, our data reproduces the fact that BMP2 reduces oligodendrogenesis in rodents and adds so far unknown information that similar to BMP7 (Baumann et al., 2015), BMP2 reduces oligodendrocyte differentiation and induces astrocyte maturation of human NPC. The differences in the published as well as in our own data concerning BMP2 effects on rodent neuronal differentiation (Table A. 25, Fig. 6b) are difficult to explain, but might be due to preparation and/or cultivation of cells in presence or absence of FCS (Brunner et al., 2010), brain region, origin of cells, plating in spheres or as single cells, co-treatment of BMP2 with or without FGF2, developmental age or species.

For mammalian NOTCH signaling, a phylogenetic very well conserved signaling pathway, activation of the transmembrane NOTCH receptors 1–4 by an extracellular ligand is crucial (Imayoshi and Kageyama, 2011). According to the transcriptome data from this study, human NPC express *NOTCH1–3*, mouse NPC all four *notch* isoforms and rat NPC also *notch1–3* and all three species display expression of different isoforms of the *NOTCH ligands DELTA-LIKE* (*DLL*) and/or *JAGGED* (*JAG*; Table A. 19). In contrast to the in silico prediction that *NOTCH* is guiding NPC migration, neuronal and glia differentiation (Fig. 5a-c), inhibition of NOTCH signaling by the NOTCH receptor inhibitor DAPT only induces neuronal and inhibits oligodendrocyte differentiation of human NPC, while it does not affect their migration (Fig. 6d-f). Although not computationally predicted as a modulator for rodent neurodevelopmental processes in our data set, DAPT reduces mouse NPC migration and induces mouse NPC oligodendrocyte formation (Fig. 6d + f), while rat NPC were not affected by NOTCH inhibition in vitro. Effects on mNPC and neuronal differentiation in hNPC were only seen at high DAPT concentrations. However, while for monolayer cultures 1 μM of DAPT showed maximum effects (Dovey et al., 2001), sensitivity towards signaling inhibitors using secondary 3D structures might be different (Alépée et al., 2014). Additionally, the specificity for Notch inhibition was shown by qRT-PCR experiments showing the expected down-regulation of HES1 and up-regulation of the pro-neuronal gene ASCL after 2.5 and 5 μM DAPT treatment (Fig. B. 7) that was not seen at the lower concentrations (data not shown). Activation of NOTCH signaling in neural stem cells (NSCs) has been implicated in inhibition of neuronal differentiation and terminal differentiation into the astrocyte lineage (Zhang et al., 2017) in several cell types and species including drosophila, early xenopus embryos, the developing chick retina, rat retinal progenitors and the developing mouse brain (Koch et al., 2013). Hence, the results obtained in the human neurospheres match the published data from other species, i.e. pharmacological inhibition of NOTCH with DAPT increases neuronal differentiation and inhibits oligodendrogenesis (Egawa et al., 2017; Nicolay et al., 2007; Zhang et al., 2017). Why mouse NPC acted in an opposite way than human NPC towards NOTCH inhibition could have several reasons. For one, in contrast to human and rat NPC, mNPC differentiation cultures contain FCS, which might be responsible for the different effects of DAPT on mouse compared to human NPC development (Brunner et al., 2010). Moreover, NOTCH favours the fate specification of oligodendrocytes and astrocytes in stages were cells are not yet committed to neuronal or glial fate, yet it inhibits the subsequent specification to $O4^+$ cells in favour of $GFAP^+$ cells (Grandbarbe et al., 2003; Park and Appel, 2003). Possible differences in developmental timing between human and mouse NPC culture might therefore provide an explanation for the different DAPT effects in human and mouse NPC. We also observed an increase in apoptotic $O4^+$ cells in the DAPT treated mouse cultures. This has previously been observed in vivo,

where transgenic mice with an inactive Notch1 receptor show premature oligodendrocyte differentiation at E17.5 which are eliminated by apoptotic cell death before full differentiation (Genoud et al., 2002). In addition it is striking, that rat NPC are not affected by NOTCH inhibition at all. This might be due to the low expression of NOTCH signaling pathway molecules (Table A. 19) keeping this pathway inactive in the neurosphere culture.

Epidermal Growth Factor (EGF)-dependent signaling regulates NSC proliferation, migration, and differentiation into neurons and glia cells during development (Ayuso-Sacido et al., 2010; Kuhn et al., 1997; Palazuelos et al., 2014; Sun et al., 2005a). Thereby, EGF exerts its action through the EGF-receptor (EGFR; Ayuso-Sacido et al., 2010; Palazuelos et al., 2014). The *EGFR* is expressed in NPC of the three species studied (Table A. 19). Investigating the effects of EGFR inhibition by PD153035 in absence of externally provided EGF gives insight into auto- or paracrine functions of endogenously produced EGFR ligands. Such ligands, like HB-EGF and TNFα, are generated through enzymatic cleavage by ADAM17/TACE (tumor necrosis factorα converting enzyme), e.g. guaranteeing survival, proliferation and development of cells of the oligodendrocyte lineage during development in an EGFR-dependent manner in mice (Palazuelos et al., 2014). The concentration of the EGFR inhibitor PD153035 selected in this study (up to 10 μM) seems high, yet different cell types display different IC50 concentrations of this EGFR inhibitor (Bos et al., 1997; Fry et al., 1994; Hsu et al., 2005). In our previous work we show that a different EGFR inhibitor, AG1478, inhibits migration and ERK1/2 phosphorylation at 1 μM (Moors et al., 2007). AG1478 and PD153035 seem equally potent in a mouse epidermal JB6 cell line (Chen et al., 2001) suggesting that effects at 10 μM concentrations observed in this study might be due to effects of PD153035 on other cellular signaling molecules. However, specificity of small signaling pathway modulators is an on-going debate as others do find specific effects of EGFR inhibitors on e.g. EGFR-expressing cancer cells at low micro molar concentrations only when cells expressed EGFR similar to non-cancer cells (Cole et al., 2005). Therefore, these data has to be interpreted with caution. Inhibition of the human NPC EGFR produced results as expected from the in silico prediction and the scientific literature, i.e. reduced NPC migration (Moors et al., 2007) and oligodendrocyte differentiation as well as induced neuronal differentiation (Ostenfeld and Svendsen, 2004; Fig. 6g-i) showing that hNPC recapitulate physiological EGFR functions in vitro. Again, *egfr* was not identified as a hub gene in rodent NPC. Yet, as expected from the literature, PD153035 increased neuronal differentiation of rat NPC (Burrows et al., 1997), while mouse NPC responded with an inhibition of neurogenesis (Fig. 6h). This discrepancy in mNPC concerning the published literature (Ayuso-Sacido et al., 2010) is possibly be due to the usage of FSC during differentiation in this study, while the C17.2 cells used by Ayuso-Sacido et al. (2010) were differentiated in absence of FCS. Studying the effects of PD153035 on human NPC differentiation in presence of FCS substantiated this hypothesis: similar to mNPC, the EGFR inhibitor reduced hNPC neuronal differentiation in presence of FCS (Fig. B. 5 m). Thus, FCS can convert cells' responses to pathway modulators implying that one has to be cautious when using FCS in cell culture medium for pathway analyses and check for human and/or in vivo relevance of data. Other than in human NPC, we did not observe inhibition of migration of rodent NPC in our study. This might be due to dissimilar EGF responses of the different glia types or maturation stages of glia in the differentiating NPC cultures of the three species (Fig. 2g; Baumann et al., 2015). Maturation and cell type-specific EGF responses due to asymmetric EGFR distribution as a mechanism for shaping brain regions or cell type diversity within brain regions was reported earlier (Sun et al., 2005b). Also in contrast to human NPC and expected from the published literature (Gonzalez-Perez et al., 2009; Hu et al., 2004; Palazuelos et al., 2014), rodent NPC oligodendrocyte differentiation is not modified by inhibition of EGFR signaling in the neurosphere cultures. Possible explanations for these discrepancies might be developmental timing and or brain region, as rat PND0–1 hippocampal NSC's differentiation to oligodendrocytes is EGF responsive, while neuronal differentiation is not (Hu et al., 2004), which is opposite in the study presented here. Another explanation could be changing EGFR levels with developmental age (Boockvar et al., 2003) in a cell type-specific manner over time (Sun et al., 2005b). It might be suggested that faster maturation of rodent NPC in comparison to human NPC determines responses to EGF.

Taken together, our transcriptome-based data demonstrates that primary NPC from different species differ in their molecular equipment despite similar cellular functions, i.e. NPC migration, neuronal and glia differentiation. Functional pathway validation due to pharmacological modulation of pathways identified via transcriptome analyses also identified species variations. Although more species-specific functional analyses of neurodevelopmental pathways need elucidation to gain a more complete picture of the human-specific NPC connectome, this work already strongly supports the concept of human cell-based in vitro analyses for neurodevelopmental toxicity or efficacy testing. Understanding such molecular pathways underlying cellular functions in in vitro systems is fundamental for understanding the assay's application domain. In addition, comprehension of similarities and differences of pathway functions between species is of high importance for pharmacology and toxicology because a high percentage of drugs fails when translating efficacy or safety from animals to humans (Waring et al., 2015).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2018.05.009.

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