

# Role of retinoids in the regulation of neural stem cell differentiation

PhD theses

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## INTRODUCTION

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The main period of neurogenesis ends prenatally, at which point, astrogliogenesis is initiated. However, active neuron formation from neural progenitors/neural stem cells continues throughout life in discrete regions of the central nervous systems (CNS) of all mammals. Stem cells are undifferentiated cells, that can develop into specialized cell types and have self-renewal capacity: they are able to go through numerous cycles of cell division while maintaining the undifferentiated state. The major sites of adult neural stem differentiation are the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ) adjacent to the lateral ventricle.

Astroglial cells are essential components of the neurogenic niches within the central nervous system. Emerging evidence suggests that they are among the key regulators of postnatal neurogenesis. For decades, astrocytes were regarded as passive elements of the brain, providing structural and metabolic support to neurons. However, a new picture about the role of astrocytes has been evolving in the recent years. Astrocytes have been shown to be involved in the regulation of the brain microenvironment, including neurotransmitter and ion homeostasis, energy metabolism, synaptic transmission and neuronal excitability, development and the maintenance of the blood-brain-barrier and the formation of the so called „stem cell niche”. A subpopulation of astroglia-type cells residing mainly within the neurogenic regions serve as neural stem cells, while the majority of non-neurogenic parenchymal astrocytes maintain the potential to support neuronal survival, growth and maturation. More recent reports have demonstrated that astrocytes induce neurogenesis both by adult neural and embryonic stem cells, *in vitro*. Although these observations confirm that astroglial cells have the potential to instruct noncommitted stem cells to adopt a neuronal fate, little is known about the factors responsible for the instructive effect.

All-trans retinoic acid (RA), a derivative of vitamin A, is one of the most powerful morphogens governing the development of various tissues and organs, including the central nervous system. During development, RA plays important regulatory roles in the formation of the neural tube and regional patterning of the future hindbrain and spinal cord. RA has been reported to regulate neuron formation both in the embryonic, as well

as in the postnatal/adult brain and has been widely used as a potent inducer of neuronal differentiation in various multipotent cell populations (embryonic carcinoma cells, embryonic and neural stem cells, induced pluripotent stem cells), in vitro.

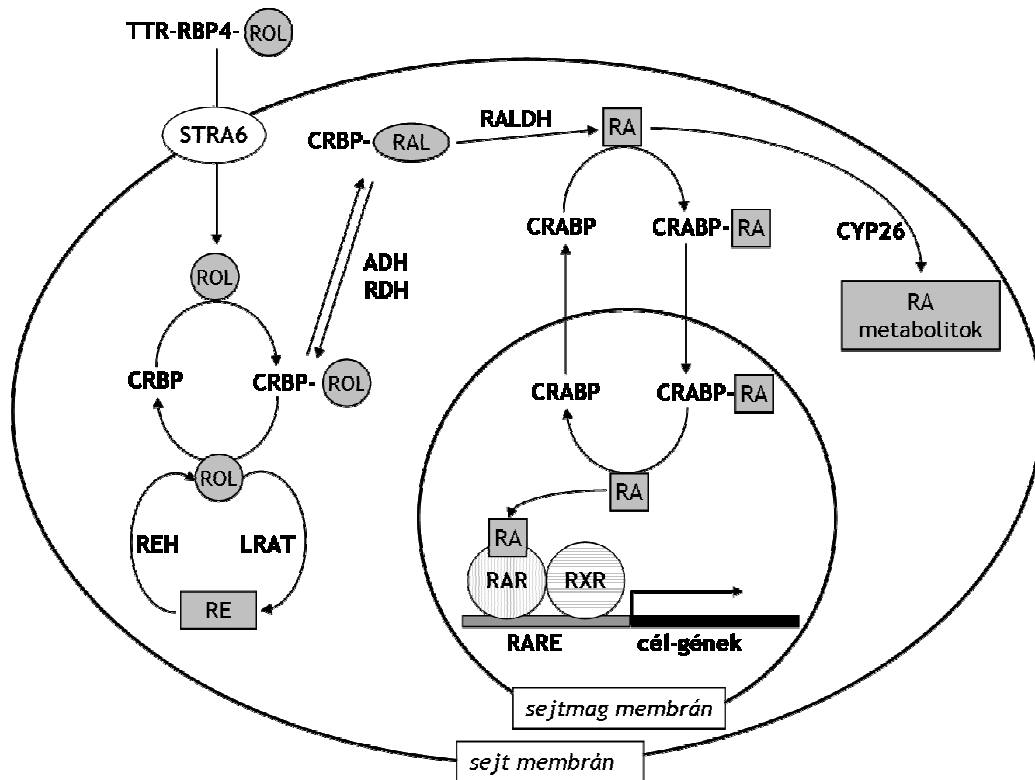


Fig.1. Retinoid metabolism. RE, retinyl ester; RAL, retinaldehyde; ROL, retinol; RA, retinoic acid; RBP4, retinol binding protein 4; STRA6, receptor for the ROL/RBP4 complex; CRBP, cellular ROL binding protein; CRABP, cellular RA binding protein; ADH, alcohol dehydrogenase; RDH, ROL dehydrogenase; RALDH, retinaldehyde dehydrogenase; REH, retinyl ester hydrolase; LRAT, lecithin ROL acyltransferase; RAR, RA receptor; RXR, retinoid X receptor; CYP26, a family of cytochrome P450 (CYP) enzymes.

The major molecular mechanisms that control RA availability and signaling were thoroughly characterized over the last decades. In brief, extracellular retinol (ROL) is delivered to the tissues via retinol binding protein 4 (RBP4), which associates with the transthyretin carrier in the circulation (Fig. 1). ROL can be taken up by cells via facilitated transport upon RBP4-ROL binding to the STRA6 transporter. Once within

the cell, ROL can either be stored after conversion to retinyl esters (RE) by lecithin retinol acyltransferase (LRAT) or it can be directed towards RA synthesis. In the latter case, ROL is reversibly converted to retinaldehyde (RAL) by ROL/alcohol dehydrogenases (RDHs/ADHs), among which RDH10 seems to play a primary role. RAL is irreversibly oxidized to all-trans RA by RALDH1-3 retinaldehyde dehydrogenases, while RALDH4 was shown to be involved in the biosynthesis of 9-cis RA. Oxidation of RA into polar metabolites is mediated by CYP26 hydroxylases, a family of cytochrome P450 enzymes. RA exerts most of its effects through activation of nuclear retinoid receptors, the heterodimers of the RA receptor (RAR) and the retinoid X receptor (RXR). All-trans RA activates RARs and 9-cis RA binds to both RARs and RXRs. The activated nuclear receptor dimers attach to RA response elements (RAREs) in the promoter regions of target genes.

The schedule of characteristic morphological changes as well as the expression pattern of neural/proneural genes was comparable in the course of retinoic acid and astroglia induced neuronal differentiation. Therefore, we hypothesized that astroglial cell induce stem cell differentiation by producing retinoic acid.

## AIMS

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The aim of my work was to investigate the role of retinoids in glia-induced neurogenesis and to study retinoid signaling and metabolism in both non-committed and differentiating stem cells. Besides, I intended to identify retinoic-acid responsive and RA producing cells within the CNS.

I intended to reveal :

- whether astrocytes are able to produce retinoic acid in vitro and if their endogenous RA production contributes to the initiation of neuronal differentiation
- whether astrocytes produce retinoic acid in vivo
- if neural stem cells are capable for endogenous retinoic acid production and retinoid utilization and is there any evidence for the autocrine regulation of neural stem cell differentiation
- what are the major cell retinoic acid responsive and retinoic acid producing cell populations in the postnatal brain

## METHODS

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### Astroglial cultures

Astrocytes were isolated from various brain regions of neonatal (P0-P2) wild-type (CD1 or FVB/N) or transgenic mice expressing eGFP under the control of the human GFAP promoter (26). In brief, meninges were removed and the tissue was minced by razor blades. The tissue pieces were subjected to enzymatic dissociation using 0,05% w/v trypsin and 0,05% w/v DNase I (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at room temperature. The cells were plated onto poly-l-lysine (pLL) –coated plastic surfaces and were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 4 mM glutamine, and 40 µg/ml gentamicin (Sigma) in humidified air atmosphere containing 5% CO<sub>2</sub> at 37°C. The culture medium was changed on the first 2 days and every second or third day afterward. Confluent primary

cultures were harvested by trypsinization and replated onto pLL-coated glass coverslips or into Petri dishes, according to the experimental design.

#### **NE-4C neuroectodermal stem cells**

The NE-4C cells are derived from the fore- and midbrain vesicles of p53-deficient 9-day-old mouse embryos and were shown to display several neural stem cell properties. The NE-4C cells were maintained in MEM supplemented with 5% FCS, 4 mM glutamine, and 40 µg/ml gentamicin in a CO<sub>2</sub> incubator at 37°C. For maintenance, subconfluent cultures were regularly split by trypsinization (0.05 w/v % trypsin in PBS) into poly-l-lysine-coated Petri dishes.

#### **Embryonic stem cell line derivation and cultivation**

The ES cell line R1 was established from (129/Sv\_129/Sv-CP)F1 3,5 day blastocyst and the CD1/EGFP ES cell line from the embryos of the TgN(GFPU)5Nagy transgenic strain. The B5/EGFP mice have been maintained on a CD1 background. RESGRO™ culture medium (THROMB-X, NV Biotechnological Development Company, Belgium) was used to obtain the CD1/EGFP ES cell line. Both R1 and CD1/EGFP ES cell lines gave high percent chimera newborns when aggregated with 8 cell-stage CD1 embryos. The newly established ES cell lines were kept on a primary embryonic mouse fibroblast feeder layer. The cells were passed in 1:4 every second day. ES cells were grown in KO-DMEM medium (Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 50 µg/ml streptomycin (Sigma), 50 U/ml penicillin (Sigma), 0,1 mM 2-mercaptoethanol (Sigma), 0,1 mM nonessential amino acids (Life Technologies), 1000 U/ml of leukemia inhibitory factor (ESGRO, Temecula, CA, USA), and 15% FCS (HyClone, Logan, UT, USA). Passages 8–10 from the newly established ES cell lines were used in our experiments.

#### **Radial glia-like cells**

Radial glia like (RGI) cells were isolated from late embryonic/fetal (E14,5–16) or adult (P50-180) mouse brains via selective attachment to adhesive surfaces covered with AK-cyclo[RGDfC] peptides. The cells were maintained in DM composed of DMEM/F12 [1:1] (Sigma-Aldrich), 1% (w/v) B27 (Invitrogen-Gibco) and 20ng/mL epidermal growth factor (EGF) (Peprotech) (EGF + ). According to the aim of the experiment, B27 supplement without (DM) or with retinyl acetate (DM+ RE) was used.

For large-scale neuron-production, EGF was withdrawn (EGF -) from the media of confluent cultures of RGI cells.

### **Astroglia/stem cell cocultures**

Briefly, in contact cocultures the stem cells were seeded on top of confluent monolayers of astroglial cells, allowing direct cell-to cell communication. In noncontact cocultures, the stem cells communicated with astrocytes only through the culture medium. The cocultures were maintained in serum-free MEM including 25% F12, 4 mM glutamine, ITS, and gentamicin (Sigma). To obtain high efficiency neuron production by ES cells, we used ES-derived embryoid bodies (EB) in combination with the astrocytes. For differentiation of the EBs, the hanging drop method was used. Briefly, a day before preparation of the hanging drops, ES cells were passaged to the gelatin (0.1%) -coated Petri dishes (Greiner, Frickenhausen, Germany) in Dulbecco's modified Eagle's medium (KO-DMEM, Life Technologies) supplemented with 15% fetal calf serum (FCS, selected batches, HyClone), glutamax (Life Technologies), and 50 mM -mercaptoethanol (Sigma). The EBs were grown in hanging drops for 24 h and subsequently plated onto astroglia monolayers in no more than 2 EB/cm<sup>2</sup> density.

### **Treatment with retinoids and RAR antagonist**

Neural stem cells were treated for varying periods with various concentrations of retinoids according to the actual experimental design. NE-4C cells were treated with 100 nM all-trans RA (Sigma-Aldrich) for an initial 48 h. In other experiments retinoids in 100 nM–10 mM concentrations were applied for 48 h or on every 2nd day. The pan RAR antagonist AGN193109 (Allergan, Inc.) was applied daily, at a final concentration of 100 nM. In experiments using retinoids, the cultures and samples were protected from light.

### **HPLC**

The astroglial cells were prepared from different areas of neonatal murine brains and maintained for 3 wk in serum containing medium. Twenty-four hours before sample collection, the cells were washed with phosphate buffer several times and 1 μM retinol containing serum-free medium was added. After the incubation period, the cells were collected for sample preparation. Sample preparation and HPLC-MS analysis were performed due to the method of Ralph Rühl. Briefly, 100 mg of the sample (if >100 mg of sample, methanol was added) was diluted with a 3-fold volume of isopropanol. The

cells were vortexed for 10 s, shaken for 6 min, and centrifuged at 13000 rpm in a Heraeus BIOFUGE Fresco at 4°C. After centrifugation, the supernatants were dried in an Eppendorf concentrator 5301 (Eppendorf, Germany). The dried extracts were resuspended with 60 µl of methanol, vortexed, shaken, diluted with 40 µl of 60 mM aqueous ammonium acetate solution, transferred into the autosampler, and analyzed.

### **Cell viability**

Different cell types grown in 96-well plates were subjected to various RAR antagonist concentrations for 48 h in serum-free medium. After the incubation period, the overall cell viability was determined according to the method by Mosmann.

### **RT-PCR analysis**

Total RNA was isolated from cells with Tri Reagent (Sigma-Aldrich) according to the manufacturer's protocol. DNA contamination was eliminated by DNase-I (Fermentas) treatment and RNA fraction was dissolved in RNase/DNase free water at a concentration of 1mg/mL. Reverse transcription reactions were undertaken from 3 mg total RNA using First Strand cDNA Synthesis Kit (Fermentas). The quantity of the cDNA product was determined by PCR (Hotstart Taq PCR Kit; Qiagen) using the hypoxanthine phosphoribosyltransferase1 (Hprt1) coding gene. For each primer pair, the PCR conditions have been optimized. The PCR products were analysed by electrophoresis on agarose gel with ethidium bromide, and were visualized by UV trans-illumination.

### **Relative quantitative real-time PCR assay**

1 mL cDNA was used for quantifying mRNA together with 300 or 900nM intron-spanning primers and 10 mL 2xPower Syber Green PCR Master Mix (Applied Biosystems). Each reaction was run in triplicate on differentiating NE-4C (DIV2, 4, 6, 10, 14), embryonic RGI or adult RGI (DIV4, DIV8) samples, as well as on nondifferentiated NE-4C or RGI (DIV0) samples. Amplification was performed in StepOne-Plus (Applied Biosystems) equipment.

### **Real-time PCR data evaluation**

Data were normalized to hypoxanthine phosphoribosyltransferase1 (Hprt1) gene expression and evaluated with StepOne Software v2.1 (Applied Biosystems) utilizing the Relative Quantitative Real-Time PCR assay evaluation with comparative CT ( $\Delta\Delta CT$ ) method. The Relative Quantity values were analyzed with one-way analysis of



variance together with the two-sided Dunnett post-hoc test to evaluate whether difference in mRNA levels of the given target between a sample and the reference of means is significant. The statistics were carried out using the SPSS for Windows 9.0 software. On the diagrams gene expression data of differentiating and undifferentiated stem cells are compared. All data are presented as Means – SD of the biological parallels and P-values below 0.1 are shown.

### **Fluorescence-activated cell sorting**

For FACS sorting experiments, we used total forebrains of P1 or P7 hGFAP-GFP transgenic mice. In older animals, the tissues were dissociated by MACS Neural Tissue Dissociation Kit (Miltenyi Biotec GmbH, Teterow, Germany) according to the protocol of the manufacturer. The cell suspensions were first gated on forward scatter, then within this population based on GFP expression. Non- GFP-expressing astrocytes were used as negative control for background fluorescence. Flow cytometry was undertaken using a FACS Vantage flow cytometry analysis system (BD Biosciences, Pers, Hungary).

### **Immunocytochemical staining**

Cultures were fixed with 4% PFA in PBS at room temperature for 20 min. Before staining, the cells were treated with 0,1% Triton X-100 in PBS for 20 min. After permeabilization, nonspecific antibody binding was blocked by incubating with 5% FCS in PBS for 1,5 h at room temperature. Primary antibodies were diluted in PBS-FCS (1:1000) and were used at 4°C, overnight. For fluorescent detection, Alexa 488 and Alexa 594 conjugated secondary antibodies were used at 1:1000 dilutions, for 60 min at room temperature. Independent experimental series and subsequent immunostainings were performed at least four times. Images were performed with Zeiss Axiovert 200M microscope or Nikon A1R confocal microscope at the IEM-HAS.

### **RA reporter bioassay**

The level of biologically active retinoids was determined by RA reporter cells. In brief, F9 embryonic carcinoma cells, carrying a RARE-lacZ construct were maintained in DMEM containing 10% FCS, in the presence of 400 mg/mL G418 (Sigma-Aldrich). F9 cells, plated 24 h before the onset of the experiments in 100,000 cell/cm<sup>2</sup> density, were treated with media conditioned by stem cells for 24 h. In other experiments, F9 cells were seeded on top of confluent layers of neural stem cells to detect the actual

retinoid production. After 18 h, the cultures were homogenized and the  $\beta$ -galactosidase activity of the homogenates was determined by the chromogenic substrate ortho-nitrophenyl- $\beta$ -D-galactopyranoside (Sigma-Aldrich). The optical density was measured at a wavelength of 410nm. The data were related to optical densities of retinoid-free cultures of the reporter cells (100%) and averages and standard deviations of relative values (as percentages) were calculated from 6–8 assays. Student's t-test was used to calculate the P-values. In experiments with NE-4C cells, the culture media were changed to defined media either lacking retinoids or supplemented with 1 mM ROL, RE or RA for the initial 48 h of differentiation. After induction, the cultures were washed several times with retinoid free medium and were subjected to the F9 reporter assay on the 8th day. RA, used in the 48 h induction period was successfully removed by the media changes and/or degraded within no more than 4 days. Radial glia like cells were maintained in defined media supplemented either with retinoid free or retinyl acetate supplemented B27 for 2 weeks before the F9 bioassays. Then the cells were differentiated by EGF removal and conditioned media were collected on the 7th day of induction.

### **Transgenic animals**

We used the RAREhsplacZ transgenic strain (Rossant et al., 1991) to study RA responsive cell populations within the postnatal CNS and hGFAP-GFP (Nolte et al., 2001) mice for cell sorting experiments.

## RESULTS

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### **Endogenous retinoic acid production by cultured astrocytes**

In this work I propose that all-trans retinoic acid, one of the major morphogenic molecules directing nervous system development, is among the astroglia-derived instructive factors. Retinoic acid is widely used as a potent inducer of neuronal differentiation by various multipotent cell populations (embryonic carcinoma cells, embryonic and neural stem cells) *in vitro*. The *in vitro* studies suggest that retinoic acid may function as a regulator of neural stem cell fate *in vivo*. This implication is supported by recent findings on retinoid signaling at sites of postnatal neurogenesis.

Retinaldehyde-dehydrogenases (Raldhs), the key enzymes for RA synthesis, convert retinal into retinoic acid. We tested the presence of the Raldh mRNAs in astroglial cells by RT-PCR. The data showed that Raldh1, Raldh2, and Raldh3 enzyme mRNAs were all present in cultured astroglial cells derived from either mesencephalon, hindbrain, hypothalamus, cerebellum, or cortex. In the lack of detailed quantitative analyses, the data obtained from a number of astroglial cultures did not provide evidence on quantitative differences in the Raldh mRNA expression between the distinct brain regions.

To evaluate the direct biological activity of glial RA production, we used a bioassay based on the retinoic acid inducible expression of the  $\beta$ -galactosidase enzyme. The  $\beta$ -galactosidase activity of the reporter F9 cells was either visualized by standard XGal staining or quantified by colorimetric assays from cell extracts using ONPG as substrate. The reporter cells responded to as low as 100 pM all-trans retinoic acid. To check whether astrocytes actively produce retinoic acid, the F9 reporter cells were plated on top of astrocytic monolayers for 18 h. Both XGal staining and colorimetric assays revealed a reliable amount of retinoic acid in the cultures.

The pan RAR antagonist AGN193109 prevented the astroglia-induced reporter cell activation. To compare the retinoic acid production of astrocytes derived from different brain regions, we used cells isolated from either hypothalami, mesencephali, cortices, cerebella, or hindbrains of postnatal (P1-P2) mice. The differences in F9 reporter cell activation by the distinct glial populations were not consistent if we compared the

results of different experimental series. Therefore, we cannot state that astrocytes from any investigated brain area produce more RAR activators than the others.

To test whether astrocytes in the early postnatal brain also produce retinoic acid, pure astroglial preparations were isolated from transgenic mice expressing eGFP under the control of the human GFAP promoter by FACS sorting. Cells sorted from newborn (P1) or 1-wk-old (P7) hGFAP-GFP mouse forebrains were seeded onto F9 reporter cells. Surprisingly, freshly isolated astrocytes did not produce retinoic acid in detectable amounts. Astrocytes isolated from P1 forebrain and cultivated for another 7 days did produce a detectable amount of retinoic acid.

The *in vivo* occurring low responsiveness of parenchymal astrocytes to retinoic acid was demonstrated by histological staining of brain sections of RARE reporter mice for  $\beta$ -galactosidase activity. GFAP-positive cells with  $\beta$ -galactosidase activity were found only sporadically in the cortex, but were relatively abundant in the lateral ventricle wall and in the hippocampus.

Retinoic acid production by cultured astrocytes was also detected when the reporter cells were grown in noncontact cocultures with the glial cells. The data indicated that astroglial cells released retinoic acid in sufficient amounts to stimulate the target cells through the culture medium. Enhanced  $\beta$ -galactosidase activity, however, was not detected when F9 reporter cells were treated with glial conditioned medium (GCM). The GCM was freshly collected after incubating astrocytes for 24 h in serum-free medium, then immediately transferred from glial cultures to the reporter cells. These data are in accord with our previous findings showing that the presence of living astroglial cells can induce neuronal differentiation by neural stem cells, whereas glial conditioned medium has no direct inductive effect.

To measure the retinoic acid content of astroglial cells, we performed HPLC analyses. All-trans retinoic acid was detected in all of the cultured astroglial populations derived from mesencephalon, cortex, cerebellum, or hindbrain. The all-trans retinoic acid concentrations varied between 3 ng/g and 6.8 ng/g, a range compatible or even wider than those found in total brain extracts by different authors. The highest all-trans retinoic acid values were measured from cortical astroglia samples. In the chromatograms of the astroglia samples, many other not yet identified peaks can be seen, indicating that other retinoid derivatives are also present in the astroglial cells.

### **Blocking retinoic acid signaling prevents astroglia-induced neuron formation**

To prevent signaling through the retinoic acid receptors, we used the pan RAR antagonist AGN193109. The stem cells were treated with the RAR antagonist for a few minutes before placing them into cocultures with astrocytes in order to prevent the immediate activation of RA-responsive genes. To make sure that the RAR antagonist itself does not affect stem cell viability, we determined the highest nontoxic concentrations of the drug.

To examine whether retinoic acid signaling was involved in astroglia-induced neuron formation, NE-4C and ES cells were treated with 100 nM RAR antagonist and put into cocultures with astrocytes. The RAR antagonist prevented the astroglia-induced neuron formation by stem cells in a concentration-dependent manner. In cocultures with astrocytes purified from different brain regions, a similar high rate decrease in neuron number could be observed. Daily treatment with the RAR antagonist, however, did not fully prevent neuron formation in any of the cocultures we tested.

As an unexpected finding, we observed ES cell-derived pulsating cell assemblies in the astroglia/ES cell cocultures treated with the RAR antagonist. The pulsating tissues were usually parts of large ES cell assemblies and were identified as heart muscle by staining for titin. The data indicate that blocking RAR signaling hinders neuronal but promotes an alternate heart muscle type differentiation.

### **RA-production by NE-4C neural stem cells and RGI progenitors**

A number of recent investigations have demonstrated that RA-responsive cells are present not only in the developing rodent brain but they also persist in the neural stem cell niches in the postnatal CNS. Retinoids are known to regulate many aspects of neural cell development, including regional and phenotype determination, gliogenesis, neurite growth, and synaptic functions. Even low (picomolar) levels of retinoic acid, however, can initiate neural development of NE-4C cells, similarly to ES cells. The presented data, however, clearly indicate that distinct neural stem cell/progenitor populations respond to retinoids in diverse ways. In NE-4C cells, despite of hardly measurable levels of retinoic acid production, retinol and retinyl acetate initiated differentiation and activated retinoic acid-responsive (Cyp26a1, Rar $\beta$ ) genes. Delays in neuron formation and gene activation suggested a rather slow accumulation of active retinoids upon retinyl acetate/ retinol induction.

In contrast to NE-4C stem cells, RGl cells produced significant amounts of RA, but displayed RA-independent differentiation program. The rate of neuronal differentiation of these cells was not affected either by the application of retinoids or RAR-antagonist. Also, RGl cells, which were prepared and maintained for several weeks in retinoid free conditions, could undergo neuronal differentiation upon EGF removal. The retinoic acid-production by RGl cells, however, seemed to depend on exogenous retinoid resources.

Both retinoid sensitivity and production by differentiating neural stem cells highly depend on the cellular composition of the cultures. In NE-4C cells, glial differentiation was negligible in the investigated period. Accordingly, the presented data reflect mainly the features of neuronal progenies of NE-4C cells. However, in RGl cells, the contribution of glial cells to the observed changes in both RA production and the retinoid machinery has to be taken into account. In mammals, many adult neural stem cells belong to GFAP-positive populations, and our adult-derived RGl clones also show GFAPimmunoreactivity. Infinite serum-free, EGF-dependent proliferation, spindle-shaped morphology and ready neuronformation upon EGF withdrawal distinguishes RGl cells from mature astrocytes. GFAP-positive, flattened astrocytes; however, appear in differentiated cultures of both embryonic and adult RGl cells. Their contribution to the retinoid metabolism can hardly be assessed.

### **Retinoic acid in the CNS**

In accordance with previous findings on retinoic acid-reporter (RARE-LacZ) transgenic mice, we identified RA-responsive cell populations in distinct, localized regions of the adult brain. These regions include the VIth cortical layers, thalamic nuclei, the hippocampus, the wall of the lateral ventricles and the meninges. RA has been shown to regulate synaptic plasticity in the hippocampus and to govern cell proliferation and neuronal differentiation in the SVZ and SGZ. In both of these regions we have observed GFAP and nestin expressing RA-responsive astrocytes, however, we could not reveal their RA producing activity. The majority of RA-responsive cells in the hippocampus was identified as granule cells in the dentate gyrus, but these cells did not express RALDH2 – one of the key enzymes responsible for RA synthesis. On the other hand, we observed strong RALDH2 expression in the choroid plexi and the meninges.

## SUMMARY OF THE RESULTS AND CONCLUSIONS

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- Astrocytes are able to induce the neuronal differentiation of different stem cell populations, in vitro.
- Astrocytes, derived from different brain regions express the mRNAs of the key enzymes of retinoic acid production. RA production of cultured astrocytes was revealed by RA-responsive bioassay and HPLC analyses.
- Blocking RA signaling results in significant reduction of neuronal differentiation in glia/stem cell co-cultures indicating that glia-derived retinoic acid is one of the major factors contributing to glia-induced neurogenesis.
- Astrocytes derived from the postnatal brain do not produce RA, though they express mRNAs of the RA synthesizing enzymes. The actual RA producing activity is likely to be upregulated in the course of in vitro maintenance.
- We have shown that both early embryonic neuroectodermal (NE-4C) stem cells and late embryonic or adult derived radial glia like progenitors (RGI cells) are capable to produce bioactive retinoids but respond differently to retinoid signals. However, while neuronal differentiation of RGI cells can not be induced by RA, neuron formation by NE-4C cells is initiated by both RA and RA-precursors (retinol or retinyl acetate). The data indicate that endogenous RA production, at least in some neural stem cell populations, may result in autocrine regulation of neuronal differentiation.
- RA is present in distinct regions of the adult brain, including the neurogenic regions, where at least some RA-responsive astrocytes were identified. The choroid plexi and the meninges showed strong RALDH2 expression, indicating their – life long - RA producing activity.

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## PUBLICATIONS

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### Publications that form the basis of the Ph.D. dissertation

Környei Z, Gócza E, Rühl R, **Orsolits B**, Vörös E, Szabó B, Vágovits B, Madarász E. (2007) Astroglia-derived retinoic acid is a key factor in glia-induced neurogenesis. *FASEB J.* 21(10):2496-509.

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**Orsolits B**, Borsy A, Madarász E, Mészáros Z, Kőhidi T, Markó K, Jelítai M, Welker E, Környei Z. (2013) Retinoid machinery in distinct neural stem cell populations with different retinoid responsiveness.

*Stem Cells Dev.* 2013 Oct 15;22(20):2777-93.

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Veszélka S, Tóth AE, Walter FR, Datki Z, Mózes E, Fülöp L, Bozsó Z, Hellinger E, Vastag M, **Orsolits B**, Környei Z, Penke B, Deli MA. (2013) Docosahexaenoic Acid Reduces Amyloid- $\beta$  Induced Toxicity in Cells of the Neurovascular Unit.

*J Alzheimers Dis.* 36(3):487-501.

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