Mechanisms regulating the formation of diverse neural pehenotypes during the *in vitro* differentiation of clonal stem cell populations

PhD thesis

Nóra Hádinger

Semmelweis University
School of Ph.D. Studies

Supervisor: Dr. Emília Madarász, DSc
IEM-HAS,
Laboratory of Cellular and Developmental Neurobiology

Opponents:	Dr. István Krizbai, PhD Dr. Nándor Nagy, PhD
Head of the Examination Committee:	Prof. András Csillag, DSc
Examination committee:	Dr. István Katona, DSc Dr. Krisztián Tárnok, PhD

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INTRODUCTION

The broad range of neurons and glial cells which build up the adult nervous system, develop from the apparently homogeneous population of neuroepithelial cells during neural development. As neural development progresses, neural tissue gets divided into morphological distinct areas among the neural axes. During development, neural progenitors produce neuronal and glial cell types determined by their location and the developmental stage. In order to produce neural cell types in precise spatial and temporal order, developmental processes need to be strictly regulated. Along the antero-posterior and dorso-ventral body axes the forming neural tissue is divided into regions which express defined sets of transcription factors. The regulatory effects of these so called “region specific transcription factors“ results in restricted activation of sub-type specific differentiation programs and leads to the production of specific neuron phenotypes. All along the neural tube, neuron production precedes asztroglia cell formation. This temporal separation of neuron and astroglia differentiation processes must be regulated by complex regulatory processes, which are not fully understood yet.

During my work I examined the retinoic acid induced *in vitro* differentiation of one cell derived murine stem cell populations (NE-4C neural stem cells, P19 teratocarcinoma cells and R1 embryonic stem cells). Understanding, how initially homogeneous stem cell populations can give rise to diverse neural cell types *in vitro*, and how we can direct stem cells to produce definite cell types, may help us to better understand the *in vivo* developmental processes, and design potential therapeutic approaches to neurological disorders.

OBJECTIVES

The major questions I was looking for answers to were the followings:

- Do NE-4C neural stem cells which are derived from an early neural developmental stage, express region specific transcription factors *in vitro*?
- What kind of region specific transcription factors get activated in course of retinoic acid induced *in vitro* neural differentiation of clonal stem cell populations?
- What kind of neurons develop during retinoic acid induced *in vitro* neural differentiation of clonal stem cell populations?
- How does the overexpression of prosencephalon specific *emx2* homeodomain transcription factor influence the phenotype and differentiation potential of NE-4C cells?
- How does *all-trans* retinoic acid (RA), known inducer of *in vitro* neuron formation, effect the process of *in vitro* astroglia formation?

METHODS

- *In vitro* differentiation of NE-4C, P19 and R1 cells was induced either with 10^{-6} M RA or by the withdrawal of FCS (fetal calf serum) or FGF2 and EGF growth factors from the culture media.
- GFP- and *emx2*-expressing NE-4C subclones were established after transfection with modified pCAGGS or *emx2*-pLenti6/V5 Directional TOPO vectors (gift from Rossella Galli - Stem Cell Research Institute, Milan, Italy) and antibiotic selection.
- Proteins were detected with immuncytochemical methods (light microscopy) at cellular, and with Western blot technique at cell culture levels.
- For gene expression analysis we used RT-PCR (reverse tanscriptase-PCR).
- In order to detect apoptotic cells we carried out Tunel (TdT-dependent dUTP-biotin nick end labelling) analysis.
- We labeled proliferating cells with BrdU (5-brome-2′-deoxyuridine).
- For detecting biologically active retinoids *in vitro*, we applied F9-RARE-LacZ reporter cell line. For *in vivo* investigations, RARE-hsp-lacZ reporter mouse strain was used.
- We blocked the nuclear receptor mediated effects of RA with pan-retinoic acid receptor (RAR) antagonist AGN193109 (10^{-7}M).

RESULTS

- **Do NE-4C neural stem cells which are derived from an early neural developmental stage, express region specific transcription factors *in vitro*?**

In our experiments, we investigated the expression of *otx2*, *emx2*, *dlx2*, *pax6*, *otx3*, *gbx2*, *hoxb2* homeodomain transcription factors. These transcription factors are expressed from the early developmental stages in defined regions of the developing central nervous system, and play important role in regionalization processes. NE-4C cells expressed only *otx2* of the investigated homeodomain transcription factors (Varga et al, 2008).

- **What kind of region specific transcription factors get activated in course of retinoic acid induced *in vitro* neural differentiation of clonal stem cell populations?**

During the RA-induced differentiation of NE-4C cells, all of the examined homeodomain transcription factors got expressed (Varga et al, 2008). We also investigated the expression of

ngn2 and *mash1* bHLH (basic helix-loop-helix) transcription factors, which are expressed mostly in complementary central nervous system (CNS) domains *in vivo*. The expression of both of *ngn2* and *mash1* got upregulated in the NE-4C cultures (Varga et al, 2008).

Similarly, homeodomain (*emx2, dlx2, hoxb2)* and bHLH *(ngn2, mash1)* transcription factors, specific for different CNS domains were expressed in P19 and R1 cultures during retinoic acid induced *in vitro* differentiation (Varga et al, 2008).

- **What kind of neurons develop during retinoic acid induced in vitro neural differentiation of clonal stem cell populations?**

In parallel with the expression of various region specific factors, NE-4C, P19 and R1 cells produced diverse neuronal phenotypes during the *in vitro* differentiation. In neuron rich NE-4C cultures, we could detect glutamatergic, GABA-ergic, serotonergic and cholinergic markers *(VGlut1, VGlut2, Gad65, Gad67, VGAT, GABA, 5-HT, chat)* at gene expression and/ or protein level (Varga et al, 2008). Despite of the expression of transcription factors (*lmx1b, phox2b* and *mash1*), playing role in specification of catecholaminergic neuronal phenotype, we could not detect catecholaminergic neuron markers *(TH, pitx3, dbh)* in the neuron rich NE-4C cultures (Varga et al, 2008). P19 and R1 cells expressed both glutamatergic *(vglut2)* and GABA-ergic *(gad65, vgat)* markers during *in vitro* differentiation (Varga et al, 2008).

- **How does the overexpression of prosencephalon specific *emx2* homeodomain transcription factor influence the phenotype and differentiation potential of NE-4C cells?**

We overexpressed *emx2*, one of the key regulators of prosencephalic regionalization processes *in vivo*, in NE-4C cells, which according to our above mentioned data are regionally uncommitted. Several NE-4C^{emx2+} clones were established. *Emx2* overexpressing cells could be stably propagated *in vitro*, and could be induced to produce neurons and astroglial cells. Although NE-4C^{emx2+} cells preserved stem cell potential, they exhibited altered phenotype compared to NE-4C "mother line". *Emx2* overexpression altered the adhesion properties of NE-4C cells. Emx2-overexpression resulted in upregulated expression of certain integrin genes and in a significant decrease of *E-cadherin* immunopositivity in NE-4C^{emx2+} cultures. Non induced NE-4C $^{emx2+}$ cells grow in a more aggregated manner than NE-4C cells. Aggregation is a first visible step of the *in vitro* differentiation of NE-4C cells, which step is

indispensable for the later neuron formation. Accordingly, neuronal markers appeared earlier in NE-4C^{emx2+} cultures compared to NE-4C cultures upon growth factor withdrawal. NE-4C^{emx2+} clones displayed upregulated expression of several markers (*egfr, hes3, blbp, pax6*) which expression was activated or enhanced only after induction of neural development in NE-4C cells.

Emx2 overexpression altered the neural stem cell phenotype. We next examined its effects on the differentiation potential of NE-4C cells. Despite the continuous expression of *emx2,* NE-4C^{emx2+} cells seemed to retain their regionally uncommitted state. Emx2 overexpression did not inhibit the expression of multiple transcription factors, those responsible for the regional specification of different CNS territories. At the time of neuron formation *in vivo, emx2* is expressed in the dorsal telencephalic ventricular zone, which gives rise to glutamatergic cortical neurons. Even so *Emx2* did not inhibit the formation of GABA-ergic or serotonine containing neurons beside glutamatergic neurons in NE-4C^{emx2+} cultures. Moreover, in contrast to NE-4C cells which failed to produce catecholaminergic cells between the given conditions (Varga et al, 2008), NE-4C^{emx2+} cells gave rise to tyrosine hydroxylase positive neurons, and expressed catecholaminergic markers in the neuron rich cultures.

- **How does *all-trans* retinoic acid (RA), known inducer of *in vitro* neuronal formation, effect the process of *in vitro* astroglia formation?**

Similarly to the *in vivo* developmental processes, astroglial cells emerged only following the main neuron forming period in course of the *in vitro* differentiation of NE-4C and P19 cells (Hadinger et al, 2009). NE-4C cells failed to generate astroglial cells during the early differentiation period even if factors that promote astroglial differentiation (*bmp2, bmp4, cntf,* neuron rich environment) were present (Hadinger et al, 2009).

All-trans retinoic acid (RA), known inducer of neuron formation both *in vivo* and *in vitro* was proposed to inhibit astroglial differentiation in the neuron forming period. Although we could not see any effect of retinoic acid on astroglial differentiation in the neuron forming stages, retinoic acid exerted influence on astroglia formation at the onset and in the astroglia forming period of the differentiation. While an initial induction with RA proved to be indispensable for the later astroglia formation, in the period of astroglia genesis, RA inhibited the appearance of GFAP (glial fibrillary acidic protein)-positive astroglial cells both in the NE-4C and P19 cultures (Hadinger et al, 2009). RA seemed to exert direct effect on astroglial

formation, as long term presence of RA does not affect the number of NE-4C derived neurons or the number of SSEA-1 positive progenitor cells persisting throughout the differentiation (Hadinger et al, 2009). RA in the applied concentration was not toxic for GFAP-positive astrocytes (Hadinger et al, 2009).

Differentiating NE-4C cells themselves produce RA. We inhibited the function of RA receptors (RAR) with pan-RAR antagonist AGN193109. If we applied AGN193109 at the early, neuron forming stages of the *in vitro* differentiation, NE-4C cells did not produce astroglial cells before schedule (Hadinger et al, 2009). Nevertheless RAR antagonist treatment significantly increased the number of GFAP-positive cells if applied after the formation of neurons in the astroglia forming period (Hadinger et al, 2009). In conclusion, endogenous RA did not delayed astrogliogenesis in the period of neuron formation, but later it regulated the number of astroglial cells generated.

CONCLUSION

- NE-4C stem cells derived from an early neural developmental stage are regionally uncommitted between *in vitro* circumstances.
- In course of *in vitro* neural differentiation of clonal stem cell populations (NE-4C neural stem cells, R1 embryonic stem cells and P19 teratocarcinoma stem cells) different region-specific genes, those expressed in different brain regions *in vivo*, got activated. Accordingly, neurons with various neurotransmitter phenotypes were generated during the *in vitro* neural differentiation.
- While NE-4C cells gave rise to glutamatergic, GABA-ergic and serotonine containing neurons, we could not detect catecholaminergic neurons in neuron rich NE-4C cultures.
- While NE-4C^{emx2+} cells retained their stem cell potential, *emx2* overexpression caused changes in stem cell phenotype. *Emx2* overexpression resulted in alterations in cell adhesion, and NE-4C^{emx2+} clones displayed upregulated expression of several genes, that were activated or enhanced in NE-4C cells only after induction of neural development. Accordingly, induction of neural differentiation evoked early appearance of neuronal markers in NE-4C^{emx2+} cultures in comparison to the NE-4C cultures. Emx2 over-expression seems to induce a transition from an early neuroepithelial stem cell state to a more developed state.

• The continuous presence of *Emx2* during in vitro neural differentiation does not prevent the expression of multiple region specific genes, those expressed in different regional patterns in vivo. Overexpression of *emx2* does not prevent the formation of GABA-ergic, or serotonine containing neurons.

• In contrast to NE-4C cells, NE-4C^{emx2+} cells gave rise to catecholaminergic neurons in the course of retinoic acid induced neural differentiation.

• Similarly to the *in vivo* developmental processes, astroglial cells emerge only following the main neuron forming period during the *in vitro* differentiation of NE-4C and P19 cells.

• Retinoic acid exerts developmental stage dependent effect on *in vitro* astroglia genesis: while it is needed for committing neural progenitors for a future production of astrocytes, it inhibits the formation of GFAP-positive astroglial cells during astroglia forming period.

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