Expression of NMDA Receptors and Ca²⁺-Impermeable AMPA Receptors Requires Neuronal Differentiation and Allows Discrimination Between Two Different Types of Neural Stem Cells

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Key Words

Neurogenesis • Neuronal differentiation • NEPs • NSCs • Retinoic acid • Ionotropic glutamate receptors • NMDA receptor • AMPA receptor

Abstract

Glutamate and its receptors are ascribed a pivotal role during activity-dependent neurogenesis. Nevertheless, their precise expression patterns during embryonic and adult differentiation remain elusive. An *in vitro*-approach that includes cells representing embryonic as well as adult neural stem cells that are both amenable to retinoic acid treatment is well-suited for assessing the developmental regulation of ionotropic glutamate receptors (iGluRs). The chosen system provides a continuous time line from embryonic to adult neurogenesis via two distinguishable cell populations, namely neuroepithelial precursors (NEPs) and radial glia-like neural stem cells (NSCs). We investigated the expression of cell type-specific differentiation markers and iGluR subunits before and after neuronal induction. A quantitative PCR assay was established for the determination of a hypothetical correlation of neuronal differentiation and iGluR

expression. The NMDAR subunits NR1 and NR2B as well as the AMPAR subunit GluR2 present in Ca²⁺-impermeable AMPARs were found to be upregulated at the mRNA level in differentiated neuroepithelial precursors, indicating their likely contribution to neurotransmission after the first establishment of neuronal networks. Furthermore, with this approach, discrimination between NEPs and NSCs regarding their iGluR subunit expression patterns before and after the induction of neuronal differentiation was possible and pointed to diverse functions in these two cell types carried out by differentially assembled iGluRs.

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Introduction

The role of the excitatory neurotransmitter glutamate and of ionotropic glutamate receptors (iGluRs) during activity-dependent neurogenesis is a hotly debated topic. Due to the high number of different receptor types and subunit combinations, the molecular diversity of

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iGluRs is large. Nevertheless, the various subunits are strictly regulated, spatially as well as temporally, thus pointing to an important role during neural development. Initial studies of the earliest glutamatergic transmission in developing neurons assumed that signalling mediated by iGluRs is solely carried out by the subfamily of N-methyl-D-aspartate receptors (NMDARs) without any contribution from alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPA) [1-4]. The activation of NMDARs is crucial, because NMDAR currents significantly contribute to the overall activity of developing circuits [5-8]. As NMDARs are blocked by Mg^{2+} at negative resting membrane potentials, they need the coactivation of AMPARs in mature neurons which serves to release the NMDARs Mg^{2+} block by prepolarization of the membrane. However, in immature neurons and neuroblasts, the required prepolarization can also be provided through the activation of excitatory gamma-aminobutyric acid A receptors (GABA_ARs). Therefore, GABA-mediated excitation during neurogenesis is indirectly responsible for the enhancement of neuronal activity and the facilitated generation of synchronized activity patterns in developing networks driven by NMDARs. Nascent synapses should only display GABA_AR-regulated NMDAR activation. This state is maintained until an appropriate synchronous activity of the pre- and postsynaptic compartment of a synapse triggers the addition of AMPARs to that synapse [1, 8]. Even though AMPARs are unlikely to contribute to early neuronal activity, several studies indicate the presence of the AMPA type of glutamate receptors even on neuronal progenitor cells [9-12]. However, observations after minimal stimulation to activate only one or few synapses revealed only NMDAR-mediated currents [4, 13], which favoured the explanation that only NMDARs were present in nascent synapses. The inability to detect AMPA-mediated currents and immunoreactivity to AMPAR subunits in nascent synapses could also be due to the fact that the signalling via AMPARs is highly sensitive to the activity state of the synapse, as AMPARs get rapidly internalized in immature synapses upon stimulation [14, 15]. Therefore, there are two possible explanations for the lack of AMPAR-mediated currents: either there are no functional AMPARs in immature neurons, or the applied high test frequencies lead to silencing of receptors. Low-frequency stimulation that is present at nascent synapses therefore would lead to a depolarization and a subsequent

activation of NMDARs. Nascent synapses thus might not be established without AMPARs, but this receptor type may be in a labile state [16].

Given the current state of knowledge, a definitive and unequivocal expression profile of iGluRs in cells of the developing CNS is much needed but unfortunately still lacking. Here, we present an *in vitro* approach to neurogenesis, which will significantly improve our knowledge about iGluR expression during neural development. The neural progenitor cells here examined, namely neuroepithelial precursors (NEPs) and neural stem cells (NSCs), represent two distinct populations of *in vitro*-generated neural stem cells. Both are able to generate neurons, but are separated on the time axis of development. They follow a defined time line of differentiation, starting from a genetically engineered embryonic stem cell line [17], which comprises a GFP and puromycin resistance gene knock-in in the open reading frame of the Sox1 gene. As Sox1 is the earliest specific marker of the neuroectoderm, this modification allows to specifically differentiate ESCs into neural precursors. It has been shown previously that both NEPs and NSCs derived from those ESCs are able to generate neurons and glia [17, 18]. NEPs are more closely related to embryonic neural stem cells, in which neuronal differentiation is more likely to occur than glial differentiation [19-22]. The opposite is true for NSCs, which already display a more astrocytic character, comparable to stem cells in later phases of neuronal development and in the adult brain [18]. Given these obvious differences, expression of differentiation markers and iGluR subunits might be differentially regulated by the induction of neuronal differentiation. Previous studies performed in our laboratory identified the molecular and electrophysiological properties of *in vitro*-generated NSCs [23]. These data were extended in the present study, in which a qPCR assay was developed which served to identify the dependence of iGluR mRNA expression on retinoic acid (RA)-mediated neuronal differentiation processes in the two different types of *in vitro*-generated neural stem cells. This study therefore provides a baseline for studies regarding the involvement of ionotropic glutamate receptors in neural development, and illuminates the first steps for further studies concerning the contribution of glutamate and its receptors to the development of the mammalian brain. Finally, our data on iGluR mRNA expression provides further clues on how to distinguish between neuroepithelial precursors and radial glia-like neural stem cells.

Materials and Methods

Stem cell culture

Cell cultures were maintained at 37°C with 5% CO₂. Neuroepithelial precursors (NEPs) were generated from 46C mouse embryonic stem cell (ESC) line obtained from E14Tg2a.IV mouse ES cells [17, 24, 25], which express EGFP under the control of the promoter of the earliest neuroepithelial marker Sox1. ESCs were passaged at a confluency of 80% and plated on gelatine-coated 3.5 cm cell culture dishes in N2B27 medium (medium composition see [17]), which promotes neuroepithelial induction. After five days, EGFP-positive, puromycin-resistant NEPs were established. EGFP-positive cells were selected by puromycin treatment (0.5 µg/ml; Sigma Aldrich) for four days.

For neuronal differentiation, cells were subsequently treated with retinoic acid (10 µM; Sigma Aldrich) for eight days.

NSCs were generated by differentiation of the genetically engineered 46C ESCs according to the protocol of Conti et al. [18] via the stage of NEPs. After generation of NSCs, their proliferative state was maintained by adding FGF-2 and EGF (both 10 ng/ml; Preprotech) to N2B27 medium. For neuronal differentiation, NSCs were cultivated for eight days in N2B27 medium supplemented with retinoic acid (10 µM; Sigma Aldrich).

RT-PCR and quantitative RT-PCR

For total RNA isolation from tissues and cultured cells, the GeneElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) was used according to the manufacturers instructions. 2 µg of total RNA was reverse-transcribed with Superscript II Reverse Transcriptase (Invitrogen). For subsequent PCRs, cDNA from approx. 50 ng of total RNA for quantitative PCR with a LightCycler Fast Start DNA Master Plus SYBR Green I kit (Roche Applied Science) was used for a single reaction. Real-time PCRs were performed on a Roche LightCycler (Roche Applied Science). Analysis was done using Roche LightCycler Software (version 3.5). Primer sequences were listed previously [23].

For relative real-time quantification, the mathematical model introduced by Michael W. Pfaffl [26] was applied. Here, the expression of the gene of interest was normalized to the expression of the housekeeping genes beta-actin and ubiquitin (2^{ΔCt}). As a positive control for constant gene expression, whole brain RNA of p2 C57/Black6 mice was used (2^{ΔCt}).

Immunocytochemistry

For immunocytochemistry, stem cell cultures were grown on polyornithin/laminin-coated coverslips and fixed in EtOH pH 2 or 4% paraformaldehyde, depending on the primary antibody used subsequently. Fixed cells were blocked either with 5% normal goat serum or 5% bovine serum albumin in PBS and stained afterwards using the following primary antibodies: mouse anti-Pax6 (DSHB; 1:50), mouse anti-nestin (Rat 401; Millipore; 1:500), goat anti-Sox2 (Santa Cruz; 1:200), rabbit anti-BLBP (Millipore; 1:300), mouse anti-vimentin (Millipore; 1:300), mouse anti-PSA-NCAM (anti-MenB [27]; 1:500), goat anti-doublecortin (Santa Cruz Biotechnology; 1:300),

mouse anti-beta-III-tubulin (Sigma Aldrich; 1:500). Secondary antibodies conjugated with Cy2 (diluted 1:250) or Cy3 (diluted 1:500) were purchased from Jackson ImmunoResearch and Dianova.

Results

Determination of the differentiation capacity of NEPs and NSCs

Amongst others, a fundamental requirement of neural stem cells is the maintenance of their undifferentiated state, which can be determined by the expression of diverse stem cell markers, as only cells, which are not yet restricted in their multipotency, retain their ability to contribute to functional neuronal networks. Therefore, we determined the stem cell state of NSCs and NEPs and their ability to differentiate into neurons. Furthermore, we addressed the question if it is possible to discriminate between NSCs and NEPs and cells differentiated from them based on their expression of ionotropic glutamate receptor mRNAs.

NEPs and NSCs express neural stem cell markers at the protein level

To determine their stem cell capacity, NEPs and NSCs were examined for their expression of EGFP (enhanced green fluorescent protein), driven by the promoter of the early neuroectodermal marker Sox1 [17, 24, 25], and a diverse panel of neural stem cell markers (Pax6, nestin, Sox2, BLBP, and vimentin) at the protein level (Fig. 1). EGFP was found to be expressed in NEPs, proving the expression of Sox1 in this cell type (Fig. 1 A). The NEP-derived NSCs have lost their Sox1 expression, indicating a more adult phenotype of this stem cell line (Fig. 1 G). Additionally, the expression of the other neural stem cell markers in NEPs and NSCs was investigated via immunocytochemical stainings. All examined markers were found to be expressed in both stem cell types (Fig. 1 B-F for NEPs, and Fig. 1 H-L for NSCs), demonstrating their undifferentiated stem cell state. Remarkably, the radial glia marker Sox2 was found to be expressed in the entire NSCs population (Fig. 1 J), whereas only a fraction of NEPs was Sox2 immunopositive (Fig. 1 D), pointing to the possibility that NSCs are more closely related to radial glia than NEPs. This finding was supported by the observation that also the radial glia markers BLBP and vimentin displayed a more thorough expression in NSCs (Fig. 1 K+L).

Fig. 1. Neural stem cell character of NEPs (A-F) and NSCs (G-L). NEPs were generated from ESCs by cultivation in a neural-inductive medium. (A) After five days of cultivation in N2B27 medium, EGFP-expressing, Sox1-positive cells emerged, which could be further selected via treatment with puromycin. (G) The expression of Sox1 is downregulated in NSCs, thus less EGFP expression can be observed. (B-F; H-L) Both NEPs and NSCs express the radial glia markers Pax6, nestin, Sox2, BLBP, and vimentin. Scale bars: 25 μ m.

Fig. 2. Differentiation potential of NEPs (A-C) and NSCs (D-F). NEPs and NSCs were treated with retinoic acid (RA) for eight days to induce neuronal differentiation. (A+D) Differentiated NEPs as well as differentiated NSCs express the early neuronal marker PSA-NCAM (polysialylated neural cell adhesion molecule), which is present on early migratory neurons. (B+E) Similarly, another early neuronal marker, doublecortin, is expressed at the protein level both in differentiated NEPs and NSCs. (C+F) More mature neurons express the neuronal marker beta-III-tubulin, a neuron-specific microtubule protein. Scale bars: 25 μ m.

Differentiated NEPs and NSCs express neuronal markers at the protein level

Next, NEPs as well as NSCs were treated with RA (10 μ M) for eight days, which is known to induce neuronal differentiation [28]. NEP colonies that formerly displayed Sox1 reactivity started to express neuronal markers. Immunocytochemical stainings with specific antibodies against PSA-NCAM (polysialylated neural cell adhesion molecule; mouse anti-PSA-NCAM; Fig. 2 A) or the neuron-specific doublecortin (goat anti-doublecortin; Fig. 2 B) and beta-III-tubulin (mouse anti-beta-III-tubulin; Fig. 2 C) used in combination with the appropriate secondary antibodies, demonstrated an abundant expression of these markers in differentiated NEPs. PSA-NCAM-positive colonies were indicators of more immature migratory

neuronal populations, while beta-III-tubulin-positive colonies comprised cells with a more mature morphology, exhibiting long neurite-like processes.

We also determined the neuronal differentiation potential of NSCs. The cells were allowed to grow to approx. 90% confluency prior to treatment with RA for eight days. They were subsequently stained immunocytochemically with antibodies against PSA-NCAM, doublecortin, and beta-III-tubulin. Differentiated NSCs exhibited immunoreactivity for the early neuronal markers PSA-NCAM and doublecortin (Fig. 2 D+E). Few of the cells were already positive for beta-III-tubulin (Fig. 2 F). Thus, immunocytochemical investigations clearly demonstrated the capacity of the two stem cell populations to effectively differentiate into neurons.

Fig. 3. Neuronal marker gene mRNA expression in NEPs and NSCs before and after RA treatment relative to the expression in mouse whole brain (postnatal day 2 = p2). The relative mRNA expression levels of the markers Pax6 (A), DCX (B), and NF-H (C) were determined by quantitative RT-PCR and normalized to the expression levels found in mouse whole brain (p2; $2^{\Delta\Delta Ct}$). Note that Pax6 (A) is most abundantly expressed in untreated NEPs, while at all other stem cell stages levels are not significantly different from those found in young mice. Concerning the neuronal marker genes, there is a progressive increase in expression from untreated NSCs to RA-treated NEPs (B+C). Data represent means $\pm$ SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant; unpaired t-test; 3-5 independent experiments.

RA treatment differentially influences marker gene expression at the mRNA level in the two stem cell types

In order to examine the differentiation progress of both neural stem cell pools before and after RA treatment compared to the expression in the control, mouse whole brain at postnatal day two (p2), mRNA expression levels of the radial glia marker Pax6, the early neuronal marker doublecortin (DCX), and the late neuronal marker neurofilament heavy chain (NF-H) were quantified (Fig. 3 A-C). We observed the following patterns: the radial glia marker Pax6 was most abundantly expressed in NEPs before RA treatment ($394.4 \pm 26.5\%$ of expression in the control; see Fig. 3 A and Tab. 1). After eight days of RA treatment, the mRNA amount of Pax6 was significantly downregulated to a level of $139.1 \pm 12.6\%$ (** $p < 0.001$), which indicated a loss of the stem cell character. In addition, the level of Pax6 mRNA in untreated NEPs was significantly higher than in the other stem cell type, namely untreated NSCs (156.7 ± 16.3 ; ** $p < 0.01$). Still, at all stages, the amount of Pax6 mRNA exceeded the expression level detected in mouse whole brain, pointing to the possibility that all cell stages are related to radial glia-like cells.

The neuronal markers DCX and NF-H were found progressively upregulated in the sequence NSCs, RA-treated NSCs, NEPs, and RA-treated NEPs (Fig. 3 B+C), indicating an increasing molecular similarity to neurons. The determination in NSCs of the mRNA amount of the early neuronal marker DCX, which is already expressed

	NSCs	NSCs + RA	NEPs	NEPs + RA
Pax6	156.7 $\pm$ 16.3	226.3 $\pm$ 77.5	394.4 $\pm$ 26.5	139.1 $\pm$ 12.6
DCX	3.2 $\pm$ 1.0	28.7 $\pm$ 1.0	72.4 $\pm$ 3.5	123.2 $\pm$ 13.7
NF-H	2.4 $\pm$ 0.8	5.4 $\pm$ 2.7	43.3 $\pm$ 9.9	75.9 $\pm$ 7.7
NR1	5.2 $\pm$ 0.9	43.4 $\pm$ 19.0	25.2 $\pm$ 7.3	198.6 $\pm$ 22.7
NR2A	120.0 $\pm$ 19.1	124.9 $\pm$ 34.6	108.5 $\pm$ 19.1	197.4 $\pm$ 36.1
NR2B	0.4 $\pm$ 0.1	2.4 $\pm$ 0.6	1.8 $\pm$ 0.3	92.9 $\pm$ 19.5
NR3A	25.9 $\pm$ 4.2	101.7 $\pm$ 33.3	19.3 $\pm$ 14.0	72.1 $\pm$ 9.1
GluR1	0.2 $\pm$ 0.04	47.8 $\pm$ 19.7	2.8 $\pm$ 2.4	83.1 $\pm$ 14.9
GluR2	4.2 $\pm$ 0.7	16.9 $\pm$ 5.2	20.6 $\pm$ 13.8	68.9 $\pm$ 20.0
GluR3	265.2 $\pm$ 26.5	79.4 $\pm$ 19.3	53.4 $\pm$ 6.3	124.7 $\pm$ 27.9
GluR4	286.2 $\pm$ 37.5	156.9 $\pm$ 70.1	60.2 $\pm$ 14.8	232.7 $\pm$ 56.7

Table 1. Relative mRNA expression levels of all examined genes compared to a mouse whole brain (p2) control. The mRNA expression levels of the individual genes were normalized to the expression of the same genes in the positive control, mouse whole brain (% of expression in the control; $2^{\Delta\Delta Ct}$). Values represent means $\pm$ SEM; n = 3-8 independent experiments.

in immature neurons, revealed an expression level of about $3.2 \pm 1.0\%$ compared to mouse whole brain (Tab. 1). This expression level was significantly upregulated after RA treatment ($28.7 \pm 1.0\%$; *** $p < 0.001$), indicating an induction of neuronal differentiation. In cultures of undifferentiated NEPs, DCX was already highly expressed ($72.4 \pm 3.5\%$ compared to mouse whole brain), but was still significantly upregulated after eight days of RA treatment ($123.2 \pm 13.7\%$; * $p < 0.05$), pointing at their neurogenic capacity. Expression level changes of the NF-H mRNA (supposed to be solely expressed in mature neurons) in untreated versus RA-treated NSCs

Fig. 4. NMDAR subunit mRNA expression in NEPs and NSCs before and after RA treatment relative to the expression in mouse whole brain (p2). The relative mRNA expression levels of NR1 (A), NR2A (B), NR2B (C), and NR3A (D) after differentiation in NSCs and NEPs compared to the expression levels in mouse whole brain (p2; $2^{\Delta\Delta Ct}$) were examined. The expression levels of the neuronal marker genes DCX (red diamonds) and NF-H (green dots) were plotted on top of the NMDAR expression levels to facilitate comparison. Note that diverging expression patterns were detectable (summarized in Tab. 2). Data represent means $\pm$ SEM; * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$; ns = not significant; unpaired t-test; 3-8 independent experiments.

showed the same tendency as for DCX, but at a very low level (2.4% and 5.4% of the control, respectively; not significant). In NEP cultures, the expression of NF-H reached $43.3 \pm 9.9\%$ of the control. This expression level was significantly upregulated (* $p < 0.05$) after RA treatment, to about $75.9 \pm 7.7\%$ of the expression in mouse whole brain.

The comparison of the two stem cell populations depicts the actual progression in the neuronal differentiation process (see Fig. 3 B+C; Tab. 1). While cultures of the early stem cell population, the NEPs, already expressed quite high levels of the neuronal markers DCX and NF-H, the expression of these markers in the later stem cell population, the NSCs, which is closely related to stem cells residing in the adult brain, was significantly lower (*** $p < 0.001$ for DCX, and * $p < 0.05$ for NF-H). This indicated a bias of NEPs to differentiate into the neuronal lineage, which is in accordance with the literature [19-22]. After eight days of RA treatment, the expression levels of DCX increased in both populations, but to a larger extent in differentiated NSCs. For RA-treated NEPs, expression of DCX doubled, while for RA-treated NSCs it increased 10-fold. The late neuronal marker NF-H was only upregulated in differentiated NEPs, indicating a complete maturation of these cells,

while in NSCs, it remained close to the detection threshold, even after RA treatment.

Ionotropic glutamate receptors show varied expression patterns dependent on the stem cell state

Considering the effects of RA treatment on neuronal marker expression in both stem cell populations analyzed, four distinct differentiation stages were determined: 1) NSCs representing the adult neural stem cells, 2) differentiating NSCs, which started to change their fate into the neuronal lineage, 3) NEPs, that were biased to differentiate into neurons, and 4) differentiated NEPs, which showed properties of differentiated neurons regarding their protein expression of neuronal markers. The expression of iGluRs was determined from the same samples that were used to determine marker gene expression.

Influence of RA treatment on NMDAR subunit mRNA expression

The mRNA expression levels of NMDAR subunits were determined in untreated and RA-treated NSCs and NEPs. RA treatment of both types of stem cells resulted in NMDAR subunit expression changes (Fig. 4 A-D;

Fig. 5. AMPAR subunit mRNA expression in NEPs and NSCs before and after RA treatment relative to the expression in mouse whole brain (p2). The relative mRNA expression levels of GluR1 (A), GluR2 (B), GluR3 (C), and GluR4 (D) after differentiation in NSCs and NEPs compared to the expression levels in mouse whole brain (p2; $2^{\Delta\Delta Ct}$) were examined. The expression levels of the neuronal marker genes DCX (red diamonds) and NF-H (green dots) were plotted on top of the AMPAR expression levels to facilitate comparison. Note that diverging expression patterns were detectable (summarized in Tab. 2). Data represent means $\pm$ SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant; unpaired t-test; 3-8 independent experiments.

Tab. 1). The mRNA amount of NR1 compared to the mouse whole brain control increased significantly after RA treatment for both stem cell types (Fig. 4 A, Tab. 1; from $5.2 \pm 0.9\%$ to $43.4 \pm 19.0\%$ after RA treatment in NSCs, ** $p < 0.01$; and from $25.2 \pm 7.3\%$ to $198.6 \pm 22.7\%$ after RA treatment in NEPs, *** $p < 0.001$). However, while the expression level of NR1 in RA-treated NSCs was still far beneath the level in mouse whole brain, the mRNA amount in RA-treated NEPs exceeded the level determined in the control. For NR2A, no significant changes were observed for either cell type after RA treatment (Fig. 4 B), indicating a minor role during differentiation. NR2B mRNA expression increased significantly in both cell types (Tab. 1; from $0.4 \pm 0.1\%$ to $2.4 \pm 0.6\%$ after RA treatment in NSCs, ** $p < 0.01$; from $1.8 \pm 0.3\%$ to $92.9 \pm 19.5\%$ after RA treatment in NEPs, ** $p < 0.01$). In RA-treated NSCs, the NR2B expression was still far from control tissue levels, while in RA-treated NEPs the expression was roughly identical to the mRNA level found in mouse whole brain, illustrating the pivotal role of NR2B in early neurogenesis, which is generally accepted [29, 30]. NR3A expression appeared to be highly dependent on RA treatment (Fig. 4 D), as its mRNA expression was also significantly upregulated in

either cell type treated with RA (Tab. 1; from $25.9 \pm 4.2\%$ to $101.7 \pm 33.3\%$ after RA treatment in NSCs, * $p < 0.05$; from $19.3 \pm 14.0\%$ to $72.1 \pm 9.1\%$ after RA treatment in NEPs, * $p < 0.05$).

We observed that compared to the expression of the neuronal markers DCX (red diamonds) and NF-H (green dots), the mRNA levels of NR1 and NR2B were dependent on the degree of neuronal differentiation indicated by neuronal marker expression, while the levels of NR2A and NR3A were not (Fig. 4). The most parsimonious interpretation is that only mature neurons express NR1 and NR2B, which are therefore likely to contribute to functional NMDARs, with NR2A playing a minor role during neurogenesis. NR3A expression seems to be independent of the type of stem cell; nevertheless, RA treatment influences its expression, so a role of NR3A during RA-mediated differentiation is likely.

Influence of RA treatment on AMPAR subunit mRNA expression

As for NMDARs, the influence of RA treatment was determined for AMPA-type iGluR mRNA levels. Again, the results are shown in relation to mouse whole brain (p2; Fig. 5 A-D). The mRNA levels of GluR1

	Neuronal marker expression-correlated	RA treatment-dependent	Stem cell type-dependent
NR1	+	+	+
NR2A	-	-	-
NR2B	++	+	+
NR3A	-	++	-
GluR1	-	++	-
GluR2	++	+	+/-
GluR3	-	+/-	+
GluR4	-	-	+

Table 2. Correlation of glutamate receptor subunit expression with the differentiation state of neural stem cell types. The expression levels of glutamate receptor subunits depend on the stem cell type as well as on the differentiation state of the cells. NR2B and GluR2 are directly correlated with the expression of the neuronal markers DCX and NF-H, while NR3A and GluR1 expression are enhanced solely by the treatment with RA, independently of the expression of DCX and NF-H, and independently of the stem cell type. Expression of NR1 is dependent on all three factors, while NR2A is dependent on none. GluR3 and GluR4 differ from the other receptor subunits, possibly due to their pivotal role in radial glia or adult neural stem cells.

changed significantly upon RA treatment in both stem cell populations (Tab. 1; from $0.2 \pm 0.04\%$ to $47.8 \pm 19.7\%$ after RA treatment in NSCs, $**p < 0.01$; from $2.8 \pm 2.4\%$ to $83.1 \pm 14.9\%$ after RA treatment in NEPs, $**p < 0.01$). In differentiated NEPs, it nearly reached the level found in the control. Thus, the expression of GluR1 was highly dependent on RA treatment, similar to what had been observed for NR3A (Tab. 2). Nevertheless, the expression also seemed to be dependent on the level of the neuronal markers, as it was approximately 35% higher in DCX- and NF-H-expressing, RA-treated NEPs than in RA-treated NSCs. GluR2 mRNA expression progressively increased during neurogenesis (Fig. 4 B). It appeared to be highly dependent on the progression of neuronal differentiation (compare red diamonds (DCX) and green dots (NF-H), Tab. 2) with only RA-treated NEPs approximating the control level (Tab. 1; $68.9 \pm 20.0\%$). GluR3 and GluR4 showed a completely different expression pattern, with the highest mRNA levels found in untreated NSCs (GluR3: $265.2 \pm 26.5\%$; GluR4: $286.2 \pm 37.5\%$), which express hardly any neuronal marker gene mRNAs (Figs. 5 C+D). These subunits have been reported to be the predominant subunits in non-neuronal cells of the brain [31, 32]. For GluR3, the expression level decreased in RA-treated NSCs to a level found in the control and did not further change significantly in either condition tested. This mRNA expression pattern indicated a functional role for GluR3 independently of other iGluRs,

probably through the involvement in radial glia or astrocytic signalling [33]. Additionally, GluR4 mRNA expression was found to peak in RA-treated NEPs ($232.7 \pm 56.7\%$ relative to mouse whole brain), underlining its specialized role early in neurogenesis, when it mediates the transport of AMPARs into silent synapses [34, 35].

We showed that eight days of RA treatment resulted in the differentiation of NSCs as well as NEPs (Figs. 4 and 5). While NEP cultures, which represent a stem cell population that has a stronger neurogenic differentiation capacity than the radial glia-like NSCs, already revealed the expression of some early neuronal markers, NSC cultures did not show any neuronal marker expression at all. RA treatment resulted in a significant increase in neuronal marker expression in both populations. Differentiated NEPs resemble the mRNA expression profile of differentiated neurons expressing AMPAR mRNA as well as NMDAR mRNA, while NSCs were only primed for the neuronal lineage.

Discussion

NSCs and NEPs represent two distinct populations of neural stem cells that are both able to generate neurons and glia, but are separated on the time axis of development. In NEPs neuronal differentiation is more likely to occur than glial differentiation [19-22]. The opposite is true for NSCs, which already display astrocytic character, comparable to stem cells in later phases of neural development and in the adult brain [36-38]. We here present evidence that the two different cell populations behave differentially also upon RA treatment *in vitro*.

RA treatment induces expression changes of cell type markers in NSCs and NEPs

RA regulates the expression of a considerable number of genes via binding to specific upstream RA-responsive elements [39], including elements that lead to neuronal differentiation. In this study, RA treatment led to changes of the mRNA amount of stem cell and differentiation markers (here examined: Pax6 as radial glia marker, and DCX and NF-H as neuronal markers) in NEPs as well as in NSCs, albeit from different starting levels. NEPs are already primed for neuronal differentiation, as revealed by the expression of the neuronal markers DCX (approx. 72% of the control) and NF-H (less prominent, 43% of the control) in untreated NEP cultures. However, expression of these genes is not

saturated as RA treatment still significantly increases the expression levels (see Fig. 3). As DCX is known to be a specific marker for immature migratory neurons [40], the abundant presence of its mRNA at the very beginning of neuronal differentiation, when newborn neurons have to migrate to their final destinations, is expected. In this phase, also markers for mature neurons start to be expressed as indicated by an increase of NF-H mRNA. In the case of NSCs, the glia-related type of stem cells, RA treatment also induced an increase in mRNA expression of neuronal markers. This increase is significant in the case of DCX, where the expression level was 10-fold increased after neuronal induction; NF-H expression was slightly, but not significantly elevated (see Fig. 3). A comparison of both populations of stem cells underscores the postulated differences between the cells: while NEP cultures already express relatively high levels of neuronal markers even prior to neuronal induction, these markers are completely absent in NSC cultures. After RA treatment of NSCs, the transcription of early neuronal genes starts, but still does not reach the starting level observed in NEPs. This expression pattern indicates that adult neurogenesis is far slower and much more complex than embryonic neurogenesis, where a highly complex organ like the brain is built in quite a short period of time. In the adult brain, new neurons are only required under certain conditions, e.g., after stroke, when huge numbers of cells have been destroyed. Under these conditions, new neurons are generated from a pool of highly proliferative progenitors, which have originated from relatively quiescent, astrocyte-like stem cells [41]. Concerning the initially astrocytic character of the stem cells, the generation of a new pool of neurons requires an event possibly related to transdifferentiation [42]. Obviously, this requires a longer time period than the direct neuronal differentiation of neuroepithelial precursors. This delay of differentiation can be observed also *in vitro*, when NSCs lose their glial identity and acquire the characteristics of neuronal progenitors, while NEPs immediately start to terminally differentiate into neurons. Concerning the relatedness to a neuronal identity, the following sequence is proposed, based on increasing expression of neuronal markers:

NSCs < RA-treated NSCs $\approx$ NEPs < RA-treated NEPs

Expression of certain iGluR mRNA requires terminal differentiation

The expression of various iGluR subunits was determined for NSCs and NEPs, and the mRNA levels

were correlated with the expression of the same genes in a control, in this case mouse whole brain tissue (p2). The observation that RA treatment leads to neuronal induction and/or differentiation was paralleled by our observation that the expression of NMDAR subunits is also influenced by RA treatment, both in NEPs and in NSCs (see Fig. 4).

Neurons generated from NEPs are likely to express NR2B-containing NMDARs, with slow deactivation kinetics and longer EPSPs. This provides the possibility to establish functional neuronal networks to stabilize neuronal connectivity [43-45]. In addition, the generation of neuronal networks in RA-treated cultures of NEPs was suggested by the morphological appearance of the cells that commenced to produce PSA-NCAM-positive, doublecortin-positive, and beta-III-tubulin-positive neurite-like processes (Fig. 2 A-C). At this point the question emerges if the developmental switch from NR2B-containing to NR2A-containing NMDARs results from an upregulation of NR2A or from a downregulation of NR2B. It is generally accepted that NR2A is expressed only later, when mature synapses are present, while NR2B levels decrease [29, 30]. In undifferentiated NEPs, NR2A mRNA is present, but as long as there is a lack of NR1 expression, it cannot contribute to functional receptors, as NR1 is needed for proper plasma membrane expression (unpublished observation). During early neuronal differentiation, NR2B mRNA gets remarkably upregulated, winning the competition with NR2A for NR1 subunits, the mRNA of which is now also expressed. While neuronal networks get established and form mature synapses, NR2A more and more contributes to NMDAR assembly, while NR2B expression declines. For a certain time, both subunits compete for the association with NR1 to build NMDARs in the postsynaptic compartment of mature neurons, but NR2A prevails over time [16, 46]. NR2B expression presumably does not vanish completely during maturation, as a certain protein level is known to contribute continuously to NMDARs. However, those receptors are essentially located extrasynaptically [46].

Assuming that the expression of the characterized markers can be correlated with the expression of NMDARs (see Tab. 2), one would expect that untreated NSCs reveal the NMDAR expression that can be found in adult stem cells (or maybe even astrocytes), while the NMDAR expression in differentiating NSCs should be similar to that found in untreated NEPs. Indeed, in RA-treated NSCs, mRNA expression of NR1, NR2A, and NR2B is not significantly different from the expression in undifferentiated NEPs, but nevertheless has changed

compared to proliferating NSCs (see Fig. 4 A-C). Furthermore, since the expression levels of NR1 are still low in differentiating NSCs, functional NR1/NR2 NMDARs are not likely to be present at this stage of development, similar to what has been found for the NEP stage.

Early glutamatergic neurotransmission is supposed to be mediated exclusively by NMDAR receptors, with little or no contribution from AMPARs [1-4]. However, other findings indicate the presence of AMPARs early in development on neuronal progenitors [9-12]. Nascent synapses are supposed to exhibit an AMPA-labile state, and the required pre-depolarization of NMDARs is thought to be carried out mainly by excitatory GABA_ARs. However, because AMPAR subunits such as GluR4 are supposed to be present on astrocytes and contribute to crucial functions like neuron-glia signalling [33], their expression might not be directly correlated to neuronal differentiation. Nevertheless, we observed that RA treatment of NEPs led to mRNA expression changes also of AMPAR subunits (Fig. 5). In NEPs, except for GluR3, all subunits investigated are significantly upregulated and reach expression levels found in mouse whole brain. This finding implicates the presence of AMPARs even during synaptic development, concomitantly with NMDAR expression. As published reports indicate a predominance of GluR2-containing, Ca²⁺-impermeable receptors involved in synaptic transmission [47, 48], it is very likely that *in vitro*-generated networks contain similar assemblies, as GluR2 expression in this study could be shown to be highly correlated with neuronal differentiation (Fig. 5 B, Tab. 2). For GluR4, a specialized role during the first postnatal week has been described [34, 35]. During this period, GluR4 is predominantly expressed on pyramidal neurons, mediating the transport of AMPARs into silent synapses, and promoting synaptic plasticity. Potential downregulation of GluR4 during maturation and its contribution to excitatory synapses remains elusive. In proliferating and differentiating NSCs, GluR3 and GluR4 levels were highly elevated, which may be explained by the astrocytic character of NSCs (as radial glia-like cells). Upon differentiation, mRNA levels of GluR1 and GluR2 increased significantly, while GluR4 remained unchanged and GluR3 levels decreased. We assume that AMPARs might contribute differentially to the formation of neuronal networks during embryonic and adult neurogenesis. Due to a low abundance of AMPAR mRNA in NEPs, AMPARs are unlikely to contribute to neuronal maturation prior to synapse formation. Differentiating NSCs already express detectable levels of AMPAR subunit mRNAs,

even though neuronal differentiation is only just being induced, as revealed by the expression of differentiation markers (Fig. 3). The GluR2 subunit is less abundantly expressed. Because GluR2-lacking AMPARs display a high Ca²⁺ permeability, they might contribute to Ca²⁺-mediated synaptic plasticity in developing neuronal networks, supporting neurogenic events such as differentiation, synaptic integration, and maturation even though NMDARs are absent.

Conclusions

A discrimination between the two stem cell types investigated, NEPs and NSCs, by means of their iGluR mRNA expression is possible and allows testable assumptions about the role of different subunits in the developing and adult brain. While mRNA expression does not necessarily predict the expression of functional proteins, the presented results only strongly suggest the presence of functional iGluRs during neuronal stem cell differentiation. Nevertheless, our findings implicate that NMDARs and AMPARs are differentially regulated during embryonic and adult neurogenesis, and that their contribution to synaptic integrity depends on the cellular character and the developmental state of the stem cell population. During embryonic neurogenesis, as exemplified by differentiation of NEPs, the first glutamatergic contribution appears to involve NMDAR activation as well as that of GluR2-containing, Ca²⁺-impermeable AMPARs; by contrast, during adult neurogenesis, exemplified by differentiation of NSCs, GluR3/GluR4-containing AMPARs are likely to play a crucial role for the proper integration of newly generated neurons into existing networks.

Abbreviations

AMPAR (Alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor); BLBP (Brain lipid-binding protein); DCX (Doublecortin); ESC (Embryonic stem cell); GABA_AR (Gamma-aminobutyric acid A receptor); iGluR (Ionotropic glutamate receptor); NEP (Neuro-epithelial precursor); NF-H (Neurofilament heavy chain); NMDAR (N-methyl-D-aspartate receptor); NSC (Neural stem cell); PFA (Paraformaldehyde); PSA-NCAM (Polysialylated neural cell adhesion molecule); qPCR (Quantitative polymerase chain reaction); RA (Retinoic acid).

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References

- 1 Durand GM, Kovalchuk Y, Konnerth A: Long-term potentiation and functional synapse induction in developing hippocampus. *Nature* 1996;381:71-75.
- 2 Gasparini S, Saviane C, Voronin LL, Cherubini E: Silent synapses in the developing hippocampus: lack of functional AMPA receptors or low probability of glutamate release? *Proc Natl Acad Sci U S A* 2000;97:9741-9746.
- 3 Isaac JT, Crair MC, Nicoll RA, Malenka RC: Silent synapses during development of thalamocortical inputs. *Neuron* 1997;18:269-280.
- 4 Wu G, Malinow R, Cline HT: Maturation of a central glutamatergic synapse. *Science* 1996;274:972-976.
- 5 Ben-Ari Y: Excitatory actions of gaba during development: the nature of the nurture. *Nat Rev Neurosci* 2002;3:728-739.
- 6 Ben-Ari Y, Khazipov R, Leinekugel X, Caillard O, Gaiarsa JL: GABAA, NMDA and AMPA receptors: a developmentally regulated 'menage a trois'. *Trends Neurosci* 1997;20:523-529.
- 7 Khazipov R, Ragozzino D, Bregestovski P: Kinetics and Mg²⁺ block of N-methyl-D-aspartate receptor channels during postnatal development of hippocampal CA3 pyramidal neurons. *Neuroscience* 1995;69:1057-1065.
- 8 Pickard L, Noel J, Henley JM, Collingridge GL, Molnar E: Developmental changes in synaptic AMPA and NMDA receptor distribution and AMPA receptor subunit composition in living hippocampal neurons. *J Neurosci* 2000;20:7922-7931.
- 9 Hagimura N, Tsuzuki K, Iino M, Takatsuru Y, Yoshida Y, Kishi S, Ozawa S: Predominant expression of GluR2 among the AMPA receptor subunits in neuronal progenitor cells of the rat hippocampus. *Brain Res Dev Brain Res* 2004;152:213-223.
- 10 LoTurco JJ, Owens DF, Heath MJ, Davis MB, Kriegstein AR: GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. *Neuron* 1995;15:1287-1298.
- 11 Maric D, Liu QY, Grant GM, Andreadis JD, Hu Q, Chang YH, Barker JL, Joseph J, Stenger DA, Ma W: Functional ionotropic glutamate receptors emerge during terminal cell division and early neuronal differentiation of rat neuroepithelial cells. *J Neurosci Res* 2000;61:652-662.
- 12 Sah DW, Ray J, Gage FH: Regulation of voltage- and ligand-gated currents in rat hippocampal progenitor cells in vitro. *J Neurobiol* 1997;32:95-110.
- 13 Rumpel S, Hatt H, Gottmann K: Silent synapses in the developing rat visual cortex: evidence for postsynaptic expression of synaptic plasticity. *J Neurosci* 1998;18:8863-8874.
- 14 Liao D, Scannevin RH, Huganir R: Activation of silent synapses by rapid activity-dependent synaptic recruitment of AMPA receptors. *J Neurosci* 2001;21:6008-6017.
- 15 Liao D, Zhang X, O'Brien R, Ehlers MD, Huganir RL: Regulation of morphological postsynaptic silent synapses in developing hippocampal neurons. *Nat Neurosci* 1999;2:37-43.
- 16 Groc L, Gustafsson B, Hanse E: AMPA signalling in nascent glutamatergic synapses: there and not there! *Trends Neurosci* 2006;29:132-139.
- 17 Ying QL, Stavridis M, Griffiths D, Li M, Smith A: Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat Biotechnol* 2003;21:183-186.
- 18 Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, Biella G, Sun Y, Sanzone S, Ying QL, Cattaneo E, Smith A: Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol* 2005;3:e283.
- 19 Haubensak W, Attardo A, Denk W, Huttner WB: Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. *Proc Natl Acad Sci USA* 2004;101:3196-3201.
- 20 Miyata T, Kawaguchi A, Okano H, Ogawa M: Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron* 2001;31:727-741.
- 21 Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR: Neurons derived from radial glial cells establish radial units in neocortex. *Nature* 2001;409:714-720.
- 22 Noctor SC, Martinez-Cerdeno V, Ivic L, Kriegstein AR: Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat Neurosci* 2004;7:136-144.
- 23 Muth-Kohne E, Terhag J, Pahl S, Werner M, Joshi I, Hollmann M: Functional excitatory GABAA receptors precede ionotropic glutamate receptors in radial glia-like neural stem cells. *Mol Cell Neurosci* 2010;43:209-221.

- 24 Aubert J, Stavridis MP, Tweedie S, O'Reilly M, Vierlinger K, Li M, Ghazal P, Pratt T, Mason JO, Roy D, Smith A: Screening for mammalian neural genes via fluorescence-activated cell sorter purification of neural precursors from Sox1-gfp knock-in mice. *Proc Natl Acad Sci U S A* 2003;100:11836-11841.
- 25 Stavridis MP, Smith AG: Neural differentiation of mouse embryonic stem cells. *Biochem Soc Trans* 2003;31:45-49.
- 26 Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29:e45.
- 27 Rougon G, Dubois C, Buckley N, Magnani JL, Zollinger W: A monoclonal antibody against meningococcus group B polysaccharides distinguishes embryonic from adult N-CAM. *J Cell Biol* 1986;103:2429-2437.
- 28 Takahashi J, Palmer TD, Gage FH: Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures. *J Neurobiol* 1999;38:65-81.
- 29 Akazawa C, Shigemoto R, Bessho Y, Nakanishi S, Mizuno N: Differential expression of five N-methyl-D-aspartate receptor subunit mRNAs in the cerebellum of developing and adult rats. *J Comp Neurol* 1994;347:150-160.
- 30 Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH: Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 1994;12:529-540.
- 31 Douyard J, Shen L, Haganir RL, Rubio ME: Differential neuronal and glial expression of GluR1 AMPA receptor subunit and the scaffolding proteins SAP97 and 4.1N during rat cerebellar development. *J Comp Neurol* 2007;502:141-156.
- 32 Riquelme R, Miralles CP, De Blas AL: Bergmann glia GABA(A) receptors concentrate on the glial processes that wrap inhibitory synapses. *J Neurosci* 2002;22:10720-10730.
- 33 Ripellino JA, Neve RL, Howe JR: Expression and heteromeric interactions of non-N-methyl-D-aspartate glutamate receptor subunits in the developing and adult cerebellum. *Neuroscience* 1998;82:485-497.
- 34 Hall BJ, Ghosh A: Regulation of AMPA receptor recruitment at developing synapses. *Trends Neurosci* 2008;31:82-89.
- 35 Zhu JJ, Esteban JA, Hayashi Y, Malinow R: Postnatal synaptic potentiation: delivery of GluR4-containing AMPA receptors by spontaneous activity. *Nat Neurosci* 2000;3:1098-1106.
- 36 Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A: Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 1999;97:703-716.
- 37 Gotz M: Glial cells generate neurons—master control within CNS regions: developmental perspectives on neural stem cells. *Neuroscientist* 2003;9:379-397.
- 38 Hartfuss E, Galli R, Heins N, Gotz M: Characterization of CNS precursor subtypes and radial glia. *Dev Biol* 2001;229:15-30.
- 39 Umesono K, Giguere V, Glass CK, Rosenfeld MG, Evans RM: Retinoic acid and thyroid hormone induce gene expression through a common responsive element. *Nature* 1988;336:262-265.
- 40 Gleeson JG, Lin PT, Flanagan LA, Walsh CA: Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. *Neuron* 1999;23:257-271.
- 41 Doetsch F: The glial identity of neural stem cells. *Nat Neurosci* 2003;6:1127-1134.
- 42 Steindler DA, Laywell ED: Astrocytes as stem cells: nomenclature, phenotype, and translation. *Glia* 2003;43:62-69.
- 43 Bear MF, Malenka RC: Synaptic plasticity: LTP and LTD. *Curr Opin Neurobiol* 1994;4:389-399.
- 44 Bliss TV, Collingridge GL: A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 1993;361:31-39.
- 45 Stevens CF, Sullivan J: Synaptic plasticity. *Curr Biol* 1998;8:R151-153.
- 46 Tovar KR, Westbrook GL: The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. *J Neurosci* 1999;19:4180-4188.
- 47 Lu W, Shi Y, Jackson AC, Bjorgan K, Doring MJ, Sprengel R, Seeburg PH, Nicoll RA: Subunit composition of synaptic AMPA receptors revealed by a single-cell genetic approach. *Neuron* 2009;62:254-268.
- 48 Seeburg PH, Higuchi M, Sprengel R: RNA editing of brain glutamate receptor channels: mechanism and physiology. *Brain Res Brain Res Rev* 1998;26:217-229.
- 49 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-685.