

Chapter 2

Directed Neuronal Differentiation of Embryonic and Adult-Derived Neurosphere Cells

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Abstract

Neurosphere-forming cells can be isolated from virtually all neural tissues during embryonic development as well as some neural tissues in the adult such as the adult subependymal zone (SEZ) and spinal cord. While these cells share in common the ability of generating clonal aggregates, the so-called neurospheres, that display self-renewal capacity and cellular multipotency (i.e., the potential to generate the three major neural lineages), they can markedly differ in their intrinsic propensity toward neurogenesis or gliogenesis as well as their ability to generate distinct types of neurons with respect to their transmitter identity. Here we discuss the endogenous differentiation potential of neurosphere cells derived from both embryonic and adult brain tissues and provide protocols how to direct adult SEZ neurosphere cells toward specific neuronal subtypes (i.e., GABAergic versus glutamatergic) via retroviral-mediated gene transfer of pro-neural genes.

Key words: Neurosphere, gene transfer, retrovirus, pro-neural genes.

1. Introduction

The neurosphere culture allows for the in vitro propagation of cells derived from various neural tissues at distinct developmental stages that share in common the potential to self-renew and to generate the three major neural cell types in the central nervous system (CNS), namely neurons, astrocytes, and oligodendrocytes (1, 2). These self-renewing, multipotent cells are often referred to as “neural stem cells (NSC)” (for critical review of the stemness of neurosphere-forming cells *see* **Chapter 1** by Louis and Reynolds). Interestingly, neurosphere-forming cells cannot only be isolated from the embryonic brain, but also persist in

some brain regions throughout adulthood and have been proposed as a potential source for providing newly generated cells for brain repair (3). The focus of the present chapter will be to provide and to discuss protocols for directing neurosphere cell differentiation toward distinct neuronal phenotypes. Understanding how neurosphere cells can be differentiated into selective neuronal types is essential when considering these cells as a source for neuronal replacement (for review *see* (4)). It also serves as a model system to study intrinsic signaling mechanisms involved in neuronal determination and differentiation *in vitro* for being highly amenable for biochemical and genetic manipulation.

Importantly, the regional and developmental origin of the neurosphere-forming cells is likely to exert a restrictive effect on their differentiation potential. This can be easily revealed by assessing the endogenous potential of neurosphere cells derived from different brain regions and developmental stages. For example, neurosphere cells derived from cells isolated from the dorsal telencephalon (i.e., cerebral cortex) at beginning of the neurogenic period (embryonic day 10 in mice) generate more neurons than neurosphere cells derived at mid-neurogenesis (embryonic day 14) from the same region (5). This reflects most likely the changes in the progenitor potential observed *in vivo* and *in vitro*: dorsal telencephalic progenitors isolated at early corticogenesis are highly neurogenic and become gradually more gliogenic at mid- to late corticogenesis (*see e.g.*, (6) and references herein). Accordingly, both neurosphere cells derived from embryonic stem cells (ESC) or embryonic telencephalic cells generate larger number of glial cells when differentiated at progressively later passages (7), suggesting that a similar transition from neurogenesis to gliogenesis has taken place in the neurospheres. Additionally, neurosphere cells keep at least some of their regional identity even after several passages (8, 9). For instance, neurosphere cells derived from embryonic dorsal telencephalon generate large numbers of glutamatergic neurons, whereas neurosphere cells isolated from ventral telencephalon or adult subependymal zone (SEZ) give rise predominantly to GABAergic cells (10, 11). Therefore, some of the original properties of stem/progenitor cells isolated from distinct germinative zones in the developing or adult brain appear to be maintained following culturing in the neurosphere assay. On the other hand, there is evidence that some transcription factors, such as Olig2, become upregulated in cells of distinct germinative zones under neurosphere growth conditions (12, 13), while other more region-specific transcription factors, such as Pax6 and Dlx2, are downregulated (13, 14). This data indicate that some intrinsic properties of the neurosphere-forming stem/progenitor cells are lost following expansion *in vitro*. Importantly, this effect may be in part attributable to culturing the neurosphere cells in high

concentrations of epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF2) (14). For instance, in the adult SEZ infusion of EGF results in the downregulation of the transcription factor *Dlx2* in rapid dividing transit amplifying cells (a type of precursor placed between the *bona fide* stem cell and fate-restricted neuroblasts), with the consequence that these cells give rise to glia at the expense of the generation of neuroblasts. This change in fate decision is consistent with the important role of *Dlx2* in adult SEZ neurogenesis (15). Given that the bulk of adult SEZ neurosphere cells during expansion in vitro divide rather rapidly thereby resembling the transit amplifying cells in vivo, EGF treatment may exert a similar effect on their fate decision. Indeed, the massive upregulation of *Olig2* that occurs in adult SEZ neurosphere cells (13) may be nothing but the other side of the coin of *Dlx2* downregulation, reflecting the switch toward a gliogenic potential (15, 16). Thus the developmental stage, tissue origin, and passage number are obviously important determining factors of the endogenous potential of neurosphere cells and hence will strongly influence the outcome of experiments that aim at analyzing the relative propensity of neurosphere cells toward neuro- or gliogenesis as well as their propensity toward generating distinct neuronal phenotypes.

Neurosphere cells can be isolated from most embryonic murine neural tissues as well as some adult murine neural tissues, including the SEZ (1), the spinal cord (17), the hippocampal dentate gyrus (18) [but possibly of a more restricted potential as compared to SEZ neurospheres (19)], the injured, but not intact, adult cerebral cortex (20), and the adult carotid body (21). Yet rather little information is available on the specific differentiation potential of these distinct types of neurosphere cells. Here, we will provide protocols focussing on how to assess the differentiation potential of neurosphere cells derived from both the embryonic cerebral cortex and the adult SEZ and will describe a methodology how to direct adult SEZ neurosphere cells toward specific neuronal subtypes via the forced expression of neurogenic fate determinants, such as the pro-neural genes *Neurog2* and *Mash1*.

1.1. Directing Neuronal Differentiation via Retrovirus-Mediated Transduction

Studies of key transcription factors acting during development have provided insights into the molecular mechanisms of neuronal subtype specification (*see e.g.*, 22–24) and provide important information with regard to the attempt to drive neural stem cells/precursor cells toward specific cell fates. It has previously been reported that the expression of pro-neural basic helix loop helix transcription factors, such as the neurogenins (*i.e.*, *Neurog2*) and *Mash1*, can effectively induce the differentiation of adult neuronal progenitors toward specific cellular fates (11, 25, 26).

Retroviral transduction is a useful method for gene delivery into neurosphere (precursors) cultures, since it is efficient and allows for stable and longlasting expression of transgenes. Non-viral gene delivery protocols such as electroporation can also be a useful method to genetically manipulate neurosphere cells. However, it has the disadvantage of being a rather aggressive method that can result in the damage of cellular integrity resulting in cell death (up to 50%). Furthermore, the introduced genes do not become faithfully transmitted to the progeny of cells continuing to proliferate and may thus become substantially diluted with each cell division. As an alternative to retroviral vectors, also lentiviral or adenoviral vectors can be exploited to efficiently transduce neural stem/precursor cells (26). One interesting difference between retroviral and other viral gene delivery methods regards the timing of gene delivery with respect to the cell division cycle as retroviral integration into the host genome requires nuclear breakdown, thus excluding nonproliferating cells from transduction. Furthermore, retroviral gene transfer may also narrow the time window for the onset of virally mediated gene expression with respect to the cell cycle. These aspects may prove important, given that the susceptibility of cells for the effect of fate determinants is very likely to depend on the cell cycle status. In the following, we will focus on the use of retroviral vectors for gene delivery into neurosphere cells, as we have most experience using this system.

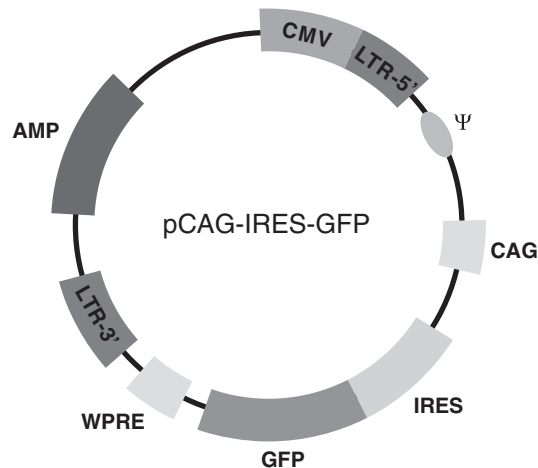
1.1.1. Retroviral Vectors

The complete cDNAs for neurogenic transcription factors such as *Neurog2*, *Mash1*, and others have been inserted into retroviral vectors previously described, such as the pCAG-IRES-GFP (27, 25), pMXIG and pCLIG (11, 28, 29, 30). These recombinant retroviral vectors contain viral long terminal repeats at their ends, a viral packaging signal, a promoter driving expression, and a gene of interest. The transgene should desirably contain a bicistronic cassette, which will allow both transgene expression and the identification of transduced cells [for example, hooking up the gene to be delivered via an internal ribosomal entry site (IRES), a nucleotide sequence that allows for translation initiation in the middle of a messenger RNA (31, 32), to enhanced green fluorescent protein (eGFP)].

The pCAG-IRES-GFP (*see* schematic map below) has been found to be highly effective in our hands, less prone to silencing compared to other retroviral vectors (pMXIG, pCLIG), likely due that the promoter sequence being an hybrid consisting of the human cytomegalovirus immediate-early enhancer and a modified chicken beta-actin promoter (33, 34). Moreover, this retroviral vector allows for increased expression of the transgene, due to the woodchuck hepatitis post-transcriptional

regulatory element (WPRE), which has been reported to stabilize transgene transcripts (35).

Schematic Map of pCAG-IRES-GFP *Schematic map of the plasmid pCAG-IRES-GFP.* The gene of interest is subcloned between the CAG promoter and the IRES-GFP. This strategy allows expression of both the gene of interest and reporter gene (for instance GFP) under control of the CAG promoter. The WPRE is located downstream of the transcript and helps to stabilize it.



1.1.2. Retroviral Genome

Simple retroviral genomes contain at least three sets of genes, *gag*, *pol*, and *env*, encoding the structural, enzymatic, and envelope proteins required for virus replication and particle assembly. In recombinant retroviral vector systems, the genes required for viral replication and particle assembly are deleted from the basic retroviral vector, with the consequence that the retrovirus is replication-incompetent and the assembly of viral particles requires *trans*-complementation of the deleted genes in a viral producer cell line. Only in cells that are either transiently or stably transfected with pro-virus plasmids (separately coding for *gag*, *pol*, and envelope proteins) can the viral particles assemble correctly containing the retroviral RNA, thus enabling it to infect cells and permanently integrate its pro-viral DNA into the host genome of dividing cells. Since the genome is split, the deleted genes have to be co-transfected into packaging cell lines, such as the human kidney cell line 293 cells (HEK293T) cells. Several alternative protocols exist regarding the mode of transfection (for instance, lipofectamin2000 or calcium phosphate transfection), the use of inducible and stably transfected packaging cell lines, such as the HEK 293-GPG cells (26, 36), and the pseudotyping using different envelope proteins [here we limit us to the use of retroviruses pseudotyped using the vesicular stomatitis virus

G protein (vsv-G), which interacts with a phospholipid component in the plasma membrane giving a rather broad host range of infection].

2. Reagents and Equipment

2.1. Culture Medium Components

1. Opti-MEM (Invitrogen, 31950-062 or 31985-070)
2. Dulbecco's modified Eagle's medium – DMEM (Invitrogen, 21969035)
3. DMEM/ F12 (Invitrogen, 31331093)
4. B27 supplement (Invitrogen, 17504044)
5. Fetal bovine serum – FBS (Invitrogen, 16140-063)
6. Antibiotics: penicillin–streptomycin (Invitrogen, 15140-122)
7. Hepes buffer, 1 M (Invitrogen, 15630)
8. 45% glucose (Sigma, G 8796)

2.2. Dissociation of Neurosphere Cells

1. Trypsin 0.05% (Invitrogen, 25300)

2.3. Glass-Coated Coverslips

1. Glass coverslips (VWR, 631-0149)
2. Poly-D-lysine (Sigma, 81358)

2.4. Culture Dishes

1. 24-well culture dishes (Becton Dickinson Labware, 3047)
2. 10 cm cell culture dish (BD Falcon, 353003)

2.5. Conical Tubes

1. 15 mL (BD Falcon)
2. 50 mL ((BD Falcon))

2.6. Filter

1. Corning® filter-top centrifuge tubes (Sigma Aldrich, CLS430320)

2.7. Plasmid DNA

1. pCMV-GP [expresses the Moloney murine leukemia virus (MoMLV) *gag-pol* genes from the immediate early promoter–enhancer of the human cytomegalovirus].
2. pCMV-VSV-G (expresses the *env* gene of the vesicular stomatitis virus, VSV)
3. pCAG-IRES GFP or pMXIG (with subcloned transgenes, e.g., *Mash1*, *Neurog2*).

Note: All plasmids and cDNAs encoding the transcription factors encoding Neurog2, Mash1 and Pax6 are available upon request from the authors.

2.8. Transfection Reagent

1. Lipofectamine 2000 (Invitrogen, 11668-019).
Note: Other transfection reagents can be used; however, lipofectamine 2000 gives consistent results with a relatively high transfection efficiency, which is prerequisite in generating viruses with a high titre. Calcium phosphate precipitation is also a relatively efficient and cheaper procedure for transfection of HEK293T cells.

2.9. Centrifuges

1. Hettich, Rotanta 460 R (for 15 and 50 mL tubes; refrigeration system)
2. Beckman coulter, Optima L-100 XP (ultracentrifuge)

2.10. Laminar Flow

1. HERA safe, KS 12 (1200×780×627 mm)

2.11. Incubator

1. VWR; BINDER, CB 210

3. Protocol

3.1. Preparing Media

	Component	For 50 mL	For 10 mL	For 5 mL
3.1.1. Differentiation Medium	DMEM/ F12	47.6 mL	9.42 mL	4.76 mL
	B27 supplement	1 mL	200 µL	100 µL
	Antibiotics	0.5 mL	100 µL	50 µL
	Hepes buffer, 1 M	0.4 mL	80 µL	40 µL
	Glucose 45%	0.5 mL	100 µL	50 µL

	Component	For 50 mL	For 10 mL	For 5 mL
3.1.2. Dissociation Medium (DMEM/10%FBS)	DMEM Glutamax	43.6 mL	8.72 mL	4.36 mL
	FBS	5 mL	1 mL	0.5 mL
	Antibiotics	0.5 mL	100 µL	50 µL
	Hepes buffer, 1 M	0.4 mL	80 µL	40 µL
	Glucose 45%	0.5 mL	100 µL	50 µL

3.2. Differentiation of Neurosphere Cultures

Overall, two methods can be used for the differentiation of neurospheres:

- (1) Whole sphere culture that allow for the identification of multipotent, bipotent, or unipotent spheres.
- (2) Dissociated cells: used to determine the relative percentage of differentiated cell types generated.

Note: A complete protocol on the preparation of neurospheres is described by Louis and Reynolds in **Chapter 1**.

3.2.1. Preparation of Glass-Coated Coverslips

Pre-coat glass slides by adding a sufficient volume of PDL to completely cover the glass coverslip for a period of 2 h at 37°C. Remove PDL and wash three times (10 min each) with sterile 0.1 M phosphate buffer saline (PBS). Remove the PBS just before plating neurosphere cells.

3.2.2. Differentiation of Whole Neurospheres

1. Typically after 7 days in vitro, floating neurospheres reach sizes of about 150–200 μm . It is not advisable to keep them longer, as further growth in size leads to cell death (probably due to low perfusion of nutrients and O_2 into the center of the sphere). Transfer the neurospheres kept in neurosphere growth medium (*see Chapter 1*) into an appropriately sized sterile tissue culture tube. Spin at 500 rpm for 2 min.
2. Remove the neurosphere growth medium and gently resuspend the neurospheres with an appropriate volume of differentiation medium.
3. Transfer the neurosphere suspension to a 10 cm Petri dish from where individual neurospheres can be visualized and harvested, using a sterile disposable plastic pipette or a Gilson P1000 pipette.
4. Transfer 3–5 neurospheres to individual wells of a 24-well tissue culture plate with PDL-coated coverslips. Add differentiation medium up to 500 μl .
5. After 7–10 days in vitro, individual attached neurospheres disperse in such a manner so as to appear as a flattened monolayer of cells.
6. Fix the cells by the addition of 4% paraformaldehyde (in PBS, pH 7.2) for 5 min at room temperature and then process the adherent cells for immunocytochemistry as required.
Notes: Neurospheres attach to the substrate quite rapidly and can be processed for immunocytochemistry after 2 h (already after this short period of time differentiating cells can be observed inside the neurospheres, indicating that neurospheres are not homogenous and comprise some fate-restricted cells). The minimum and maximum times for differentiation need to be tested for each application. For example, it may be necessary to culture the cells under differentiation conditions for longer periods of time (up to a month) to obtain fully functional neurons. On the other hand, 3 days might be enough to assess for multipotentiality of neurosphere-forming cells (**Fig. 2.1**).

3.2.3. Differentiation of Dissociated Cells

1. Typically after 7 days in vitro, floating neurospheres reach sizes of about 150–200 μm . As stated above, it is not advisable to expand them for longer time. Transfer the neurospheres kept in the neurosphere growth medium into

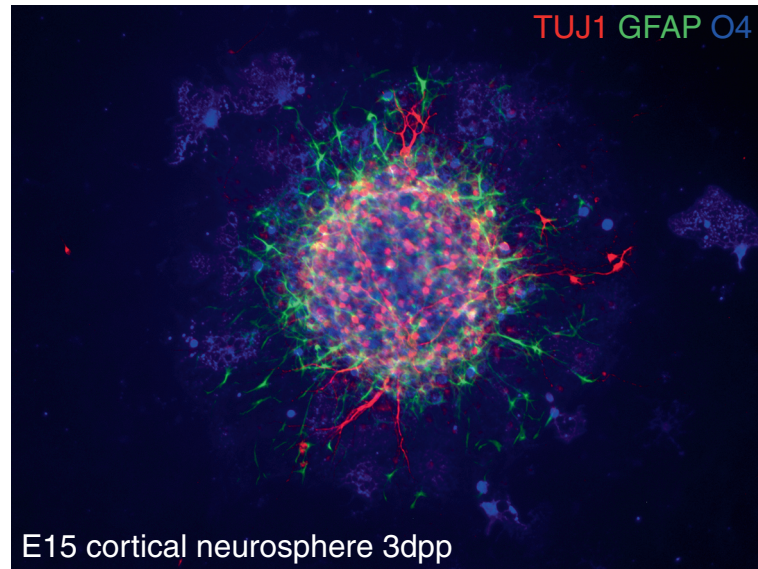


Fig. 2.1. Neurosphere cells express cell type-specific markers shortly after differentiation. The micrograph depicts a whole neurosphere grown from E15 cortical cells that was plated under adherent conditions without EGF and FGF2. Three days post-plating (dpp), neurons, astrocytes, and oligodendrocytes can be distinguished by immunocytochemistry using antibodies against class β III-tubulin (Tuj1), glial fibrillary acidic protein (GFAP) or O4, respectively. Note that cells have migrated little out from the neurosphere at that stage.

- an appropriately sized sterile tissue culture tube. Spin at 500 rpm for 2 min.
2. Remove the neurosphere growth medium and add 1 mL trypsin 0.05%. Gently mix and incubate at 37°C for 5 min.
3. Add 1 mL of DMEM/10% FCS and dissociate neurospheres mechanically by using a fire-polished Pasteur pipette. Centrifuge the cell suspension(s) at 1000 rpm for 5 min.
4. Remove the neurosphere growth medium and then gently resuspend the cells with 1–2 mL of DMEM/10% FCS. Centrifuge the cell suspension(s) at 1000 rpm for 5 min. Repeat this step to completely remove trypsin from the cell solution.
5. Remove the neurosphere growth medium and then gently resuspend the neurospheres with an appropriate volume of differentiation medium.
6. Combine an aliquot from the cell suspension with trypan blue and count live cells.
7. Prepare the appropriate cell suspension in differentiation medium so as to seed individual wells of 24-well tissue culture plate containing a PDL-coated glass coverslip with $1-2 \times 10^5$ cells (Final volume 500 μ L).

8. After 7–10 days in vitro, most cells will have differentiated sufficiently to be characterized via immunocytochemistry using antibodies against neuronal and glial antigens.
9. Fix the cells by the addition of 4% paraformaldehyde (in PBS, pH 7.2) for 5 min at room temperature and then process the adherent cells for immunocytochemistry as required.

3.2.4. Viral Infection of Whole and Dissociated Neurosphere Cultures

1. After plating, cells can be infected with viruses carrying a bicistronic cassette encoding for the protein of interest and a reporter gene, such as green or red fluorescent proteins (GFP and RFP, respectively) for visualization. For retroviruses, transduction needs to be performed preferentially shortly after plating as the cells have to undergo mitosis to incorporate the retroviral vector before inducing differentiation. For lentiviral gene delivery, infection can be performed at any time, as both proliferating and postmitotic cells will be transduced.

Note: Viral infection must be performed under particular safety conditions in accordance with legal regulations. The medium containing pro-viral particles must be replaced/ discarded in accordance with these regulations.

2. Choosing the appropriate viral delivery system: The choice between retro- or lentiviral expression of a given gene can markedly affect the outcome of the experiment. In contrast to lentiviral vectors, retroviral vectors will integrate only into genomes of dividing cells. This means that expression driven by the construct will commence with specific timing with respect to the cell cycle which is likely to affect the functional consequences of expression of fate determinants. Our experience suggests that neurogenic fate determinants encoded by retroviral vectors are more efficient in driving adult neurosphere cells toward neurogenesis than the same genes encoded by lentiviral vectors.
3. Choosing the amount of virus to be used for transduction: Depending on the experiment, it might be desirable to obtain different numbers of transduced cells. The amount of virus used to transduce cells will be then determined by two main factors: (i) how many cells the experimenter wants to infect; this will vary depending on the experimental question: for instance, if a clonal analysis should be performed, it is desirable to infect only few clones per cover slip to avoid that nearby clones become infected simultaneously, thus suggesting the wrong interpretation of being one single large clone; (ii) the titre of the original viral stock solution (*see below*). Additionally, for retroviral vectors the proportion of transduced cells will also depend on the proportion of cells undergoing cell division.

3.2.5. Virus Production

This method is modified from previously described method (37). The aim is to produce a relatively large batch of highly concentrated virus by harvesting the medium from twelve 10 cm plates. However, depending on the transgene and retroviral backbone (size and other unknown factors), the number of plates required for the same quantity of virus produced will vary.

1. Unthaw HEK293T cells at 37°C and plate 5 million (5×10^6) HEK293T cells in 5 mL Opti-MEM per 10 cm Petri dish.

Note: HEK293T cells should be maintained as a stock using standard cell culture methods in DMEM with 10% FBS.

2. At 24 h after plating, add 3 mL of the DNA/lipofectamine¹ solution onto each Petri dish of 293T cell plate. Transfer dropwise while rocking the Petri dish. Incubate at 37°C for 5–6 h.
3. Replace medium with fresh DMEM/ 10% FBS in order to wash out DNA/lipofectamine solution. Incubate 48 h at 37°C.
4. Collect the supernatant of medium in 50 mL Falcon tubes (2 days after transfection).
5. Filter the supernatant through 0.22 µm filter top and transfer filtered supernatant into ultracentrifuge tubes.
6. Centrifuge at 50,000*g* at 4°C using an ultracentrifuge for 2 h.
7. Remove the supernatant with a Pasteur pipette.
8. Resuspend the precipitated virus with 1 mL Opti-MEM by pipetting up and down. Pool the resuspended viruses and fill up tubes with Opti-MEM.
9. Repeat the centrifugation step at 50,000*g* at 4°C using an ultracentrifuge for 2 h.

¹ *Troubleshooting: Preparing the DNA/lipofectamine solution:*

- (A) Mix Opti-MEM and cDNAs (pro-viral cDNAs and backbone plasmid containing your gene of interest). Incubate at room temperature for 5 min (*Solution A*).
For each 10 cm Petri dish:
9 µg of retroviral back bone DNA (e.g., pCAG-IRES GFP or pMXIG with transgene)
6 µg of CMV-GP
3 µg of CMV-VSV-G
1.5 mL of Opti-MEM
- (B) Mix Opti-MEM and lipofectamine 2000. Incubate at room temperature for 5 min (*Solution B*).
For each 10 cm Petri dish:
60 µL lipofectamine 2000
1.5 mL of Opti-MEM
- (C) Mix *Solution A* and *B* by constant pipetting up and down and tapping the tube. Incubate at room temperature for 30 min.

10. Remove the supernatant with Pasteur pipette.
 11. Resuspend the final pellet in a desired volume of Optimem or PBS, (recommended 50–100 μL for the first time). Let the pellet incubate in medium for 2 h at 4°C.
 12. Pipette up and down until pellet is dissolved. Aliquot in small volumes as viruses do not tolerate repeated refreezing. Store at –80°C until use.
- Note:** It is possible to harvest viruses, from the same cells transfected, a second and third time, by replacing the medium with new medium (Opti-MEM) and repeat the harvesting procedure 2–3 days later (Steps 4–12).

3.2.6. Determining the Viral Titre

Note: Determining the titre of the virus should ideally be done in the cell type used for the experimental question, as the infection rate is dependent on cell cycle kinetics and the general infectivity of cells. For simplicity, however, titration may be performed in a cell line. Here we describe determining the titre using HEK293T cells.

1. Plate 30,000 HEK293T cells per well in 24-well plate on coverslips. Add 500 μL of DMEM/10%FBS and incubate at 37°C. Seed six wells for each virus preparation.
2. Perform a serial dilution of viruses (example: mix 199 μL of medium with 1 μL of viral stock). Transfer 100, 20, 10, 2, 1, and 0.2 μL of mixture to each well. These volumes correspond to 1/2, 1/10, 1/20, 1/100, 1/200, and 1/1000 of the viral stock. The denominator of these fractions is termed *Diluting Factor (DF)*.
3. After 48 h, fix the cells by the addition of 4% paraformaldehyde (in PBS, pH 7.2) for 5 min at room temperature and then process the adherent cells for immunocytochemistry as required (for example, using anti-GFP primary antibody followed by a secondary antibody conjugated to a fluorescent protein, in order to amplify the signal for the endogenous GFP produced in a cell transduced with pCAG-IRES-GFP).
Note: In some cases, the endogenous levels of fluorescent reporter proteins are high enough to allow direct visualization after fixation. However, we recommend the staining to amplify the signal always, because there might be cells expressing lower, undetectable levels of fluorescent reporter proteins and, as a consequence, the titre will be underestimated.
4. Count the number of fluorescent colonies (clusters of cells) in wells where there are not too many infected cells (i.e., per coverslip with less than 100 colonies). The virus titre per millilitre is calculated by the formula:

$$\text{Number of colonies } (n) \times \text{dilution factor } (DF) \times 10^3$$

Example: If you count 50 colonies in a well where 2 μL of the viral mixture (200 μL medium + 1 μL viral stock) had been added:

$$\text{Titre} = 50(n) \times 100(\text{DF}) \times 10^3 = 5 \times 10^6 \text{ viral particles/ml}$$

Note: From viral supernatants obtained from 12 plates resuspended in a volume of 50–100 μL , one should obtain titres of 10^7 – 10^8 viral particles per millilitre.

4. Typical Protocol Results

4.1. Neurospheres Derived from Developing Cortex Generate Glutamatergic Neurons

According to the literature when neurospheres derived from mouse embryonic dorsal telencephalon are differentiated, distinct proportions of neurons, astrocytes, and oligodendrocytes are generated, depending on (1) the age when cells were obtained (5) and (2) the number of passages prior to differentiation (7). We have observed that neurons derived from E14 embryonic cortical neurospheres acquire a predominantly glutamatergic identity, in contrast to neurospheres derived from embryonic ventral telencephalon (10) or adult SEZ (11). **Figure 2.2** shows examples of neurons derived from E14 cortical neurospheres. Note that virtually all MAP2-positive neurons also express Tbr1 (**Fig. 2.2A,B**), a transcription factor involved in the specification of glutamatergic neurons (38). Electrophysiological pair recording of neurons in these cultures confirmed their glutamatergic phenotype, as revealed by the complete block of the postsynaptic responses in the presence of CNQX, a competitive AMPA/kainate receptor antagonist (**Fig. 2.3**). We have also observed the majority of

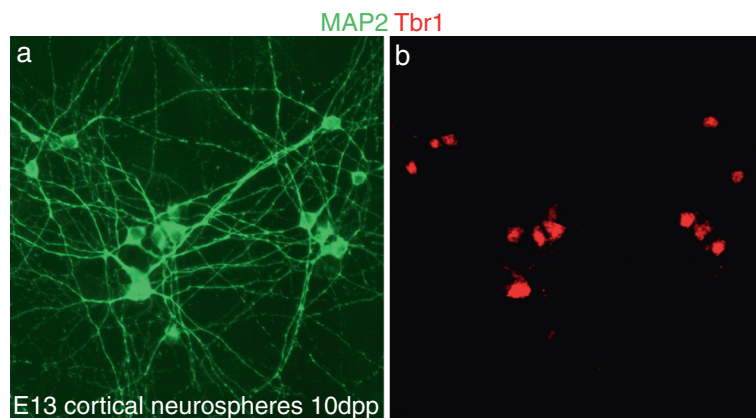


Fig. 2.2. Embryonic cortical neurospheres generate glutamatergic neurons. **(A)** The micrograph depicts a dissociated neurosphere cell culture 10 dpp, stained for the dendritic marker protein MAP2. **(B)** The micrograph depicts the same culture stained for Tbr1, a transcription factor involved in the genesis of glutamatergic neurons. Note that virtually all MAP2-positive neurons also express Tbr1.

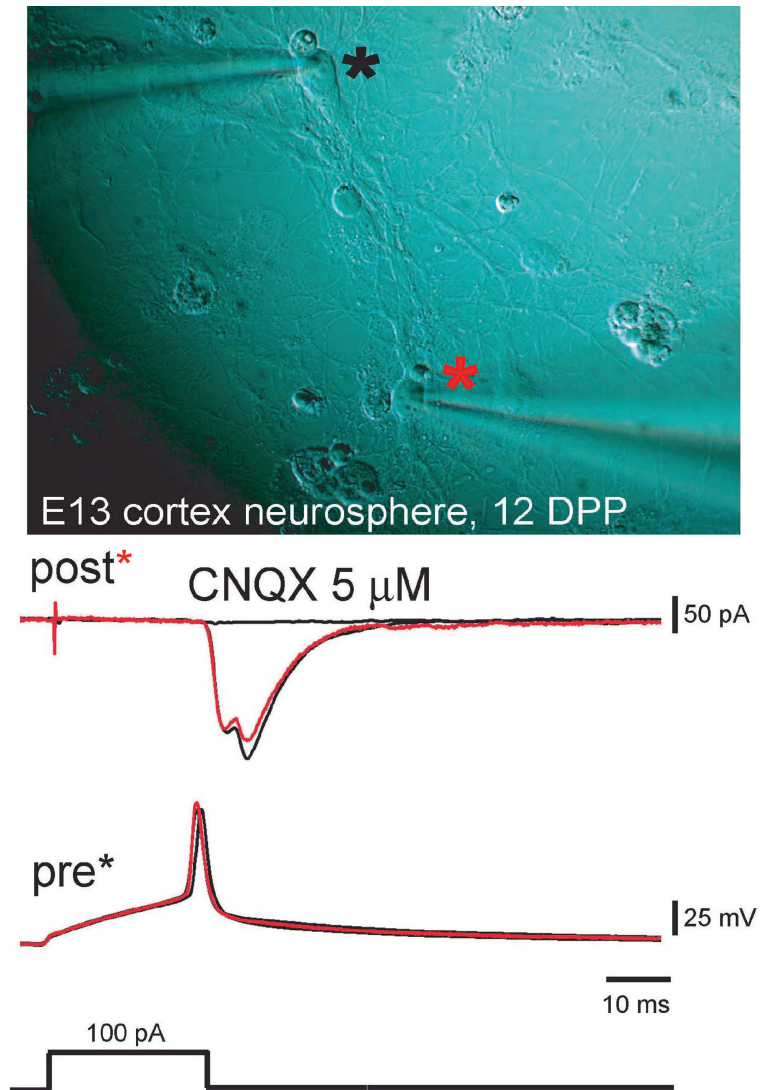


Fig. 2.3. E13 cortical neurosphere cells give rise to functional glutamatergic neurons. (A) Bright-field micrograph showing two neurons derived from E13 cerebral cortical neurospheres, 12 days post-plating (dpp). The neuron marked with the asterisk is presynaptic (pre* in b) to the other neuron (post in b). (B) In current clamp, a brief step current injection of 100 pA induces an action potential in the presynaptic neuron (pre*). The postsynaptic neuron (held in voltage clamp) exhibits a synaptic response (black trace), which is blocked by the AMPA/kainate receptor antagonist CNQX, indicating the glutamatergic nature of the presynaptic neuron. Following washout (red trace), the response returns to the control level. The response exhibits a short-latency monosynaptic and a later polysynaptic component, presumably due to recruitment of another glutamatergic neuron synapsing onto the postsynaptic neuron.

MAP2-positive neurons derived from E14 cortex neurospheres express a vesicular glutamate transporter (vGluT) up to the fifth passage (data not shown), indicating that although the overall neurogenic potential of cortical neurosphere cells becomes

progressively reduced with passage number, the neurochemical properties of the neurons generated appear still to be consistent with the regional identity from which the original neurosphere-forming cell had been isolated.

4.2. Directing Adult SEZ Neurosphere Cells Toward Specific Transmitter Identities

4.2.1. Endogenous Potential of Adult SEZ Neurosphere Cells

When allowed to differentiate, adult SEZ neurosphere cells generate a certain proportion of neurons (roughly between 5 and 15%). The transmitter identity of these cells is heterogeneous: Approximately 80–90% of all neurons adopt a GABAergic identity as shown by their expression of GFP when isolated from GAD67::GFP knock-in mice, in which GFP is driven by the promoter of the GABA-synthesizing enzyme isoform glutamic acid decarboxylase (GAD) 67 (not shown). The remaining cells exhibit vesicular glutamate transporter (vGluT) immunoreactivity (11). These data are consistent with the electrophysiological analysis, which reveals that the majority of cells form GABAergic synapses and autapses (synaptic connections of a neurons onto itself), but there is a subpopulation (i.e., roughly 10–20%) of glutamatergic neurons within this culture system (11). **Figure 2.4** shows an example of such a glutamatergic neuron derived from an adult SEZ neurosphere cell. Presumably, the differences in transmitter identity are accounted for by the mosaic nature of

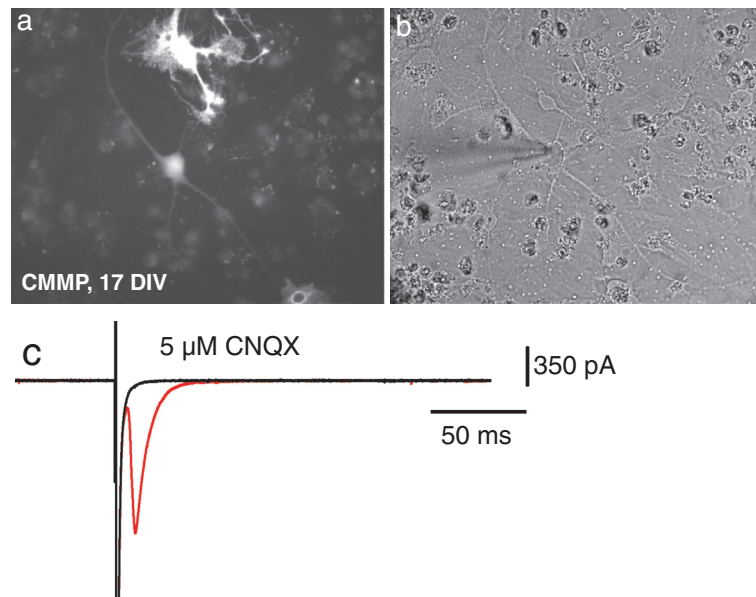


Fig. 2.4. A small percentage of control vector (CMMP encoding GFP) transduced adult SEZ neurosphere cells generate glutamatergic neurons. (A) Fluorescence micrograph depicting a GFP-positive neuron close to a GFP-positive astrocyte, 16 days post-infection (dpi). (B) Bright-field micrograph depicting the recorded cell. (C) Step depolarization of the neuron in voltage clamp resulted in the activation of an autaptic connection (i.e., connection of the stimulated neuron onto itself; *red trace*) that was entirely blocked by the AMPA-type glutamate receptor blocker CNQX (*black trace*).

the adult SEZ (39). There is growing evidence that there is not a single generic neural stem cell but distinct “stem cells” that differ with respect to their potential for subtype specification and hence transmitter identity. A still open question in this regard is whether neurons derived from the same neurosphere (that is of clonal origin) can acquire distinct neurotransmitter identities or whether all neurons derived from the same neurosphere-forming cell acquire the same neuronal phenotype.

4.2.2. Forced Expression of *Neurog2* Drives Adult SEZ Neurosphere Cells Toward a Glutamatergic Identity

In order to direct adult SEZ neurospheres toward specific neuronal phenotypes, we have previously shown that expression of neurogenic fate determinants not only favors neurogenesis at the expense of gliogenesis but also permits the acquisition of a specific transmitter identity (11). Transcription factor-driven differentiation of neurosphere cells may be a valuable tool for understanding the molecular mechanism underlying subtype specification as considerable numbers of cells can be generated, thereby permitting a biochemical analysis.

Following transduction with the proneural gene *Neurog2*, >95% of the neurosphere cells adopt a neuronal identity (26, 11). Moreover, when analyzing their phenotype, these neurons exhibit the feature of typical glutamatergic telencephalic neurons (**Fig. 2.5**). This is consistent with the fact that within the developing telencephalon *Neurog2* is expressed in the dorsal domain, which gives rise to glutamatergic pyramidal neurons (40). Morphologically, *Neurog2*-transduced neurosphere cells resemble cultured pyramidal neurons (**Fig. 2.5**) and over time

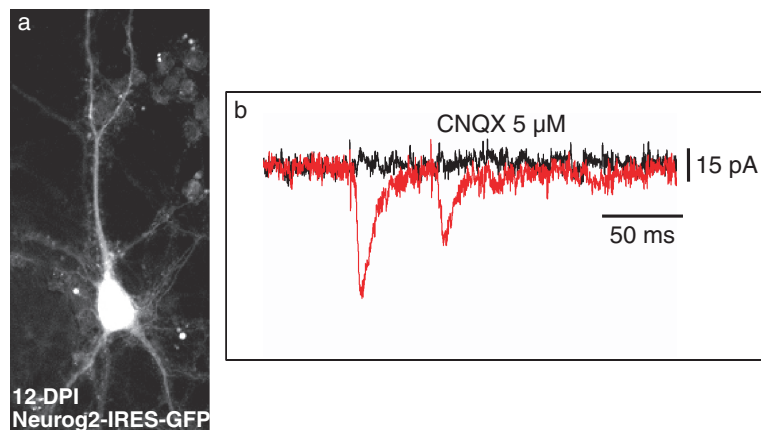


Fig. 2.5. *Neurog2*-transduced adult SEZ neurosphere cells give rise to glutamatergic neurons. **(A)** Fluorescence micrograph depicting a *Neurog2*-expressing neuron after 12 days post-infection (dpi). The neuron exhibited a pyramidal neuron-like morphology. **(B)** Dual recording from the cell depicted in **(A)** and a neuron nearby revealed that activation of the *Neurog2*-expressing neurosphere cell generated a postsynaptic response (lighter trace) in the other neuron which was blocked by CNQX (Black trace), indicating its glutamatergic nature.

in culture acquire dendritic spines. Massive expression of vGluT immunoreactivity (11) indicates that virtually all transduced cells have acquired a glutamatergic identity, suggesting a quantitative effect of Neurog2 expression. Conversely, there is no expression of GABAergic markers (such as GABA and GAD) in neurons derived from Neurog2-expressing neurosphere cells, indicating that these cells do not acquire a hybrid identity. Consistent with their neurochemical features, Neurog2-transduced neurosphere cells also form glutamatergic synapses and autapses (**Fig. 2.5**). Electrophysiological signs for the presence of synapses can be obtained around day 9 post-infection, indicating that the maturation of these cells does not occur at a slower pace compared to murine cortical neurons in culture. Particularly interesting is the fact that following Neurog2 expression there is also upregulation of the downstream transcription factor Tbr1 (**Fig. 2.6**), which is known to form part of the transcriptional cascade involved in specification of glutamatergic neurons (38). However, in contrast to E13 neurosphere cultures (example above), Tbr1 expression is detected in only one third of the transduced cells (**Fig. 2.6**). One explanation may be that not all Neurog2-transduced neurosphere cells acquire the same subtype specification. However, given the wide spread expression of vGluT and our evidence from electrophysiological recordings that virtually all Neurog2-expressing neurosphere cells become glutamatergic neurons, the limited expression of Tbr1 may indicate that this transcription factor is expressed only transiently. Alternatively, expression may be restricted to a subpopulation of Neurog2-transduced cells with additional subtype characteristics. For instance, while Tbr1 is believed to be expressed in virtually all glutamatergic neurons in the developing cerebral cortex, its expression is maintained only in a subpopulation of deep-layer neurons (38). These findings point to the interesting possibility that expression of neurogenic fate determinants may permit to direct not only the neu-

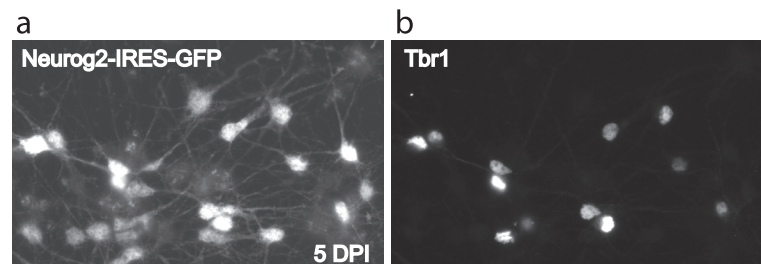


Fig. 2.6. Induction of the T-box transcription factor Tbr1 following forced expression of Neurog2 in adult SEZ neurosphere cells. **(A)** Fluorescence micrograph depicting a culture transduced with a retrovirus encoding Neurog2-IRES-GFP 5 days post-infection (dpi). The transduced cells all exhibited a neuronal morphology. **(B)** Many of the transduced cells also express Tbr1.

rotransmitter identity of adult SEZ cells but also their precise subtype. Using this approach, it may be in principle possible to selectively generate different classes of cortical neurons (i.e., callosal, corticothalamic, or subcortical projection neurons) in vitro. Future studies will have to show whether by expression of additional fate determinants in conjunction with *Neurog2*, such as the zinc finger protein *Fez1* (41), a more precise subtype specification can be achieved.

4.2.3. Forced Expression of *Mash1* Promotes a GABAergic Identity

Given the fact that the close relative of *Neurog2*, *Mash1*, is expressed in the developing ventral telencephalon where mostly GABAergic neurons are generated (40), we hypothesized that forced expression of *Mash1* may limit the differentiation potential of adult SEZ neurons to a GABAergic fate. Indeed, we found that virtually all neurons expressing *Mash1* adopt a GABAergic phenotype (11) (**Fig. 2.7**). However, neurons derived from *Mash1*-expressing neurosphere cells mature more slowly compared to *Neurog2*-expressing cells (for instance, with regard to the pace of synapse formation). Thus, while principally confirming the approach, other or additional factors may be needed to differentiate adult SEZ into selective types of GABAergic neurons. Recently, we have shown that adult SEZ neurosphere cells can be

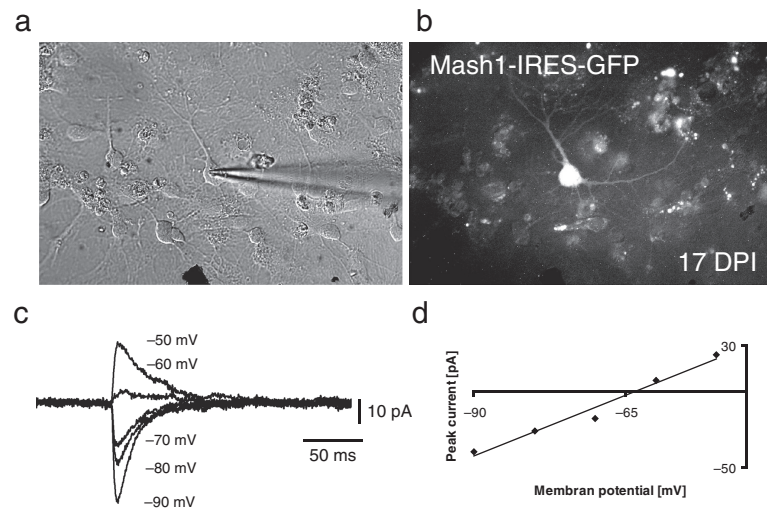


Fig. 2.7. *Mash1* expressing adult SEZ neurosphere cells give rise to GABAergic neurons. **(A)** Bright-field micrograph depicting a culture 17 days post-infection (dpi). **(B)** Fluorescence micrograph depicting a *Mash1*-expressing neuron derived from an adult SEZ neurosphere. **(C)** Step depolarization of the *Mash1*-expressing neuron resulted in a post-synaptic response in a nearby neuron held at different holding membrane potential to reveal the reversal potential of this synaptic connection. The response reversed already at membrane potentials more positive than -65 mV, indicating that it is of inhibitory (i.e., GABAergic) nature. **(D)** The peak current of the synaptic response is blotted versus the holding membrane potential of the postsynaptic neuron.

driven toward neurogenesis by forced expression of *Dlx2*, another transcription factor in the genesis of GABAergic neurons (15). It remains to be shown whether *Dlx2* or other transcription factors promote the specific generation of GABAergic neurons.

5. Future Directions

The selective differentiation of adult neurosphere cells as exemplified here by the forced expression of *Neurog2* and *Mash1* may suggest that it should be in principle possible to selectively direct neurosphere cells toward distinct neuronal sublineages. As discussed above, by combining expression of factors inducing a generic glutamatergic fate (such as *Neurog2*) in conjunction with transcription factors responsible for the specification of additional features such as precise location within the circuitry (e.g., layer specificity in case of cortical neurons) and projection patterns (e.g., callosal versus subcortical), it may become feasible to tailor adult SEZ-derived neurosphere cells into specific types of neurons that degenerate in different neurodegenerative diseases, as for instance corticospinal motor neurons that are lost in amyotrophic lateral sclerosis. A challenge for the future will be to test whether neurosphere cells from the adult SEZ can also be differentiated toward neuron subtypes occurring in non-telencephalic brain regions, such as midbrain dopaminergic neurons that degenerate in Parkinson's disease. This may bear therapeutic implications, but also provide insights into the fate limitations of these adult "stem cells." If it turns out that the site of origin from where the neurosphere-forming cells have been isolated poses limits on the plasticity of their progeny, it may become even more important to assess the differentiation potential of neurosphere cells derived from other regions such as the spinal cord (17).

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