An optimized experimental strategy for efficient conversion of embryonic stem (ES)-derived mouse neural stem (NS) cells into a nearly homogeneous mature neuronal population

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A B S T R A C T

NS cells are a homogeneous population of neural stem cells which were previously derived from embryonic stem cells as well as from the fetal and adult brain. Our previous reports have described a 21 day long neuronal differentiation protocol able to reproducibly convert adult SVZ-derived NS (aNS) cells into a population composed of 65% mature neurons and 35% glial cells. Here we have developed a different procedure specifically applicable to ES-derived NS cells in order to fully explore their neurogenic capacity. Differently from the aNS differentiation procedure, optimized neuronal output from ES-derived NS cells requires replating of the cells on appropriate substrates followed by sequential exposure to modiﬁed media. In these conditions, ES-derived NS cells differentiate into neurons with a barely appreciable quota of astrocytes and occasional oligodendrocytes. In particular, 21 days after the beginning of the treatment, 85% of the cells has differentiated into molecularly and electrophysiologically mature neurons belonging to the GABAergic lineage. The procedure, which is applicable with no considerable differences to different ES-derived NS cell lines and to NS cells at different passages, opens to the possibility of molecular and biochemical studies on close-to-uniform stem cell derived neurons.

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Introduction

During the last decades many efforts have been devoted to the isolation and reliable expansion of neural stem cells with a particular emphasis to the development of protocols that would allow their differentiation in vitro into functionally mature neurons. Accomplishments in these directions would represent an important milestone in stem cell biology and a crucial starting point for biochemical, toxicological and pharmacological studies (Gottlieb, 2002).

To this aim, a number of important protocols have been set up for the differentiation of embryonic stem (ES) cells into neurons. However, the reported differentiation achieved by these procedures is not synchronous nor uniform, leading to the coexistence in the culture of differentiated neurons and non-neural cells together with neural precursors and undifferentiated ES cells (Pollard et al., 2006a, b; Ying et al., 2003). A notable exception is represented by a study by Bibel and co-workers showing that homogeneous cultures of cortical glutamatergic neurons can be efﬁciently generated from ES cells using an optimized embryoid bodies based protocol (Bibel et al., 2004).

In parallel, a number of protocols have been developed which were focussed on in vitro derivation of mature neurons from stem cells taken directly from brain tissue, also as a consequence of the vigorous push given by the definition of the conditions for the isolation and growth of neural stem cells. These achievements have been accomplished through immortalization of the target neural stem/progenitor cells or via their expansion as neurosphere cultures. Nevertheless, in both cases one of the impediments is represented by the poor neuronal differentiation potential of these cells both in vitro and following intracerebral transplantation. Protocols for neurosphere differentiation in vitro generally consist of sphere attachment on treated substrate followed by mitogen removal (Conti et al., 2005; Conti et al., 2006; Vescovi et al., 1993). Initial protocols suggested to plate cells from early passaged neurospheres onto polyornithine-coated coverslips in the absence of growth factors for 5 days, followed by the addition of fetal bovine serum (FBS) for an additional 2 to 5 days (Vescovi et al., 1993). In these conditions, however, the identity
of the stem cells was not clear and less than 15% of MAP2 immunopositive neurons were found which could be also eventually partially derived from the coexisting neuroblasts in the neurosphere culture (Gritti et al., 1999). Alternative protocols have been subsequently developed but overall neuronal differentiation efficiency never was more than 20% (Grandbarbe et al., 2003; Weiss et al., 1996; Garcia et al., 2004; Tropepe et al., 1999). A step forward is represented by the study by Hack et al. (2004). These authors reported that Pax6 overexpression by mean of retroviral transduction in neurosphere cultures resulted in a marked increase of neuronal differentiation efficiency with the fraction of β3-tubulin immunoreactive raising from 25% in the control cultures to 85% in the Pax6 overexpressing cultures. However, this study was performed on early passaged neurospheres (passage 4) and it is likely that the genetic modification has acted on already committed cells rather than on stem cells. In addition, characterization of the neuronal features acquired by the differentiated cells was limited to β3-tubulin immunoreactivity.

Altogether, this evidence highlights that the neurogenic potential of short-term and long-term passaged neurosphere-derived cells, in spite of the extensive work performed by several groups, has never been improved beyond what described above.

In 2005 and subsequent years, some of the current authors reported the derivation of adherently growing, self-renewing murine neural stem cells from ES cells and primary tissues obtained from the fetal and adult nervous system (CNS) (Conti et al., 2005; Goffredo et al., 2008; Pollard et al., 2006a, b). These cells, which have been named ‘NS’ (Neural Stem) cells, grow in monolayer in a defined medium while resembling the neurogenic radial glia of the brain, although its transient nature in vivo constitutes a first difference with respect to the unlimited self-renewing capacity of cultured NS cells. Both ES-derived and adult tissue-derived NS cells have been proven to give rise to differentiated cells belonging to all three mature lineages of the CNS, i.e. astrocytic, neuronal and oligodendroglial (Conti et al., 2005; Glaser et al., 2007; Goffredo et al., 2008). In particular, the original protocol for neuronal differentiation described in 2005 indicated that ES-derived NS cells are able to differentiate into antigenically and electrophysiologically mature neurons and retain this competence after prolonged in vitro expansion (Biella et al., 2007; Conti et al., 2005). Although these results are unequivocally valuable in these conditions the total number of surviving differentiated cells is low (between 5 and 10% of the plated cells after 21 days in vitro) making thus problematic the exploitation of these stem cell derivatives for molecular and biochemical studies as well as drug screening purposes.

Here we report a substantial improvement in the originally described protocol for neuronal differentiation of ES-derived NS cells. This procedure shows to be proficient both in terms of efficiency (more than 80% of the differentiating cells are mature GABAergic neurons after 21 days in vitro), survival rate (more than 70% of the plated cells survive after 21 days in vitro) and reproducibility. This procedure generates undistinguishable results even after prolonged in vitro expansion of the differentiating ES-derived NS cells. The efficiency of this protocol in term of production of neurons from stem cells is the highest ever reported for unmodified cells. Taken together, our results lead us to believe that both this method and its products might constitute a useful tool for investigations of stem cell biology and genetic and chemical screens in stem cells and their neuronal progeny.

**Materials and methods**

**Cell lines**

The LC1 and LC1-EGFP cell lines were derived from the 46C ES cell line as previously described (Conti et al., 2005). In this study, LC1-EGFP cells have been passaged continuously in vitro for over 130 passages without gross changes in their proliferation and differentiation patterns. Other NS cell lines employed in this study were NS-CGR8 and NS-R1, derived from ES cell lines CGR8 and R1, respectively (Conti et al., 2005). NS cells are routinely grown in uncoated 25 cm² flasks (Iwaki, Barworld) in Euromed-N medium (Euroclone, Cellbio) supplemented with 1% N2 (Gibco, Invitrogen) and EGF and FGF-2 (20 ng/mL each; PeproTech, Tebu-Bio) (Conti et al., 2005). Cultures are generally split 1:4 to 1:5 every 3–4 days. Cells are regularly passaged by Accutase (Sigma) dissociation (2 min at 37 °C). Cells are pelleted by centrifugation (3 min at 1,200 rpm). Every 3 days the medium is changed with fresh one.

**Optimized neuronal differentiation procedure**

The procedure for terminally differentiating ES-derived NS cells here described was entirely developed under serum-free conditions. The neuronal differentiation process was optimized using 3 steps-specific media as schematically indicated (Fig. 2A). During all these steps the cells are maintained in serum-free conditions (media composition is detailed in Supplementary Table 1). Step 1: almost confluent (80–90% density) cultures are dissociated for 1 min using Accutase, and the cell suspension was centrifuged at 1000 g for 3 min. The pellet is resuspended and the cells were counted and plated (density: 1.0 × 10⁶–1.5 × 10⁶ cells/cm²) on uncoated plastic cell culture flask-dish using D1 medium consisting of EUROMED-N medium (Euroclone, Cellbio) supplemented with 1% B27 (Invitrogen), 0.5% N2 (Invitrogen) and 10 ng/mL FGF-2 (Peprotech, Tebu-Bio). Cells are maintained in these conditions for three days at 37 °C. Step 2: cells are gently dissociated with Accutase and spin at 1000 g for 3 min. The pellet is resuspended by gentle pipetting and the cells were plated (density: 5 × 10⁴–7.5 × 10⁴ cells/cm²) on laminin coated (3 μg/mL for 3–5 h at 37 °C) plastic or glass coverslip in freshly made pre-warmed medium A composed of a 1:3 mix of DMEM/F12 and Neurobasal media (Invitrogen) containing 0.5% N2 and 1% B27 supplements and containing FGF-2 (10 ng/mL) and BDNF (20 ng/mL) for 3 days. Step 3: cells are shifted to medium B composed of a 1:3 mix of DMEM/F12 and Neurobasal media containing 0.5% N2 and 1% B27 supplements and containing FGF-2 (6.7 ng/mL), and BDNF (30 ng/mL). After three days, medium B was replaced with fresh medium B with a reduced amount of FGF-2 (5 ng/mL). The cells were maintained under these conditions for an additional 10–15 days; the medium was partially changed every 3 days.

**Immunocytochemistry**

Self renewing and differentiating NS cell cultures were fixed in cold 4% PFA (paraformaldehyde) for 15 min at room temperature. After washing in Phosphate Buffered Saline (PBS), the cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min and subsequently blocked with 4% normal goat serum in PBS for 1 h. Primary antibodies were incubated for 16 h at 4 °C in PBS + 4% normal goat serum. A list of primary antibodies used in this work is presented in Supplementary Table 1. At the end of the incubation with primary antibody and after washing in PBS, the cells were incubated with appropriate secondary antibodies (Alexa Fluor 488 or 555, 1:500, Molecular Probes, Invitrogen). Finally, the nuclei were counterstained with Hoechst 33258 (5 μg/mL; Molecular Probes, Invitrogen) and the samples were mounted with Permaflor (Immunon, Italscientifica). The images were acquired with Axiovert 200 M (Zeiss) and a DM6000 (Leica) microscope. For quantitative analyses, we counted 300–500 cells per well and the values were averaged from quadruplicate samples in at least three independent experiments.

**Expression profiling and data analysis**

For this study LC1-EGFP cells at passage 30 were employed. RNA was harvested using the RNasy columns with additional DNase step
using the manufacturers protocol (Qiagen). Microarrays used for expression profiling were the Murine 430A Genechip arrays (Affymetrix). RNA quality controls (Agilent Bioanalyser), cDNA synthesis, cRNA probe preparation and hybridization were performed by GeneService Ltd (Cambridge, UK). Normalized data from the Affymetrix software were analysed using the GeneSifter microarray data analysis system (VizX Labs LLC, Seattle, WA). Briefly, data was pooled before analysis and then RMA normalization applied to the pooled data. As part of the RMA procedure the data was normalized using a non-linear (quantile) method. This analysis was combined with an average fold change analysis. All analysis was performed on log transformed data. Genes were selected as candidate markers if 1.5-fold higher in D1 treated NS cells compared to self-renewing NS cells.

Western blot assay

Cells and tissues were lysed by adding 100 μl of SDS sample buffer (62.5 mM Tris–HCl pH 8.5, 2% SDS, 10% glycerol, 50 mM DTT and 0.1% bromophenol blue). The lysates were subject to shearing by passage through a 20-gauge (0.9 mm) needle. Samples were heated at 95–100 °C for 5 min and then cooled on ice. Proteins (10 μg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide running gel. Gels were blotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked by incubation for 1 h with 5% non-fat powdered milk in Tris-buffered saline containing 0.2% Tween 20 (TBS-T) and then rinsed with TBS-T. Protein blots were incubated with the primary antibodies diluted in 3% non-fat powdered milk in TBS-T. For information about working dilutions and suppliers, see Supplementary Table 2. After overnight incubation at 4 °C, blots were washed with TBS-T and then treated with horseradish-peroxidase conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Bio-Rad), diluted 1:2000 in TBS-T containing 3% non-fat powdered milk, for 1 h at room temperature. After 4 washes with TBS-T, protein bands were detected by ECL kit (Amersham). Western blot quantification analysis was performed with Quantity One software (Bio-Rad).

Electrophysiology

Solutions for electrophysiological recording

A cell between the electrodes and cells were established in a bath solution containing (in mM): 155 NaCl, 1 CaCl2, 1 MgCl2, 3 KCl, and 10 Hepes/NaOH (pH 7.4). To avoid contamination of voltage responses with low threshold Ca2+ spikes, the bath solution contained also the Ca2+ channel blocker NiCl2 (1 mM) when required. After the whole-cell configuration had been established, the pipette filling solution for current-clamp and total ionic current recordings in voltage-clamp was combined with an average fold change analysis. All analysis was performed on log transformed data. Genes were selected as candidate markers if 1.5-fold higher in D1 treated NS cells compared to self-renewing NS cells. When the filling solution for current-clamp and total ionic current recordings in voltage-clamp was contained (in mM): 128 KCl, 11 EGTA, and 10 Hepes/KOH (pH 7.04).

The patch pipettes were made of borosilicate glass tubing (Hilgenberg GmbH, Malsfeld, Germany) and fire polished to a final resistance of 0.5–2 MΩ when filled with internal solution. All of the experiments were performed at room temperature (22–24 °C). The whole-cell currents were recorded using an Axopatch 200A amplifier (Axon Instruments Inc., Burlingame, CA), digitized at sampling intervals of 20 μs (voltage-clamp) and 200 μs (current-clamp) using a Digidata 1322A AD/DA converter (Axon Instrument Inc.). Stimulation, acquisition, and data analysis were carried out using PCLAMP (Axon Instrument Inc.) and Origin software (Microcal Software Inc., Northampton, MA, USA). Fast capacitive transients were reduced online using analog circuitry, and residual capacitive and leak currents were removed by P/4 subtraction. The currents were filtered at 5 kHz.

Results

Definition of an optimized in vitro protocol for the efficient neuronal commitment of ES-derived NS cells

Our previous studies indicated that ES-derived NS cells are able to generate electrophysiologically mature neurons after 3–4 weeks of exposure to neuronal differentiation protocols (Conti et al., 2005). However, these protocols generate only a limited number of neurons that can be maintained for longer than 10 days in vitro. These results prompted us to investigate whether this condition represents the maximal competence of the system to generate neurons or whether it reflects a limit of our differentiation procedures, thus underlying the requirement to precisely optimize both the survival and the generation of mature neurons. To address this issue, we have worked towards the definition of an optimized procedure in order to obtain large amounts of enriched populations of functional neurons from short-term and long-term expanded ES-derived NS cells. In the beginning, we focussed our attention onto two critical steps of neuronal differentiation, i.e. the neuronal commitment and the neuronal maturation. The neuronal commitment step consists of instructing a neural progenitor to choose a neuronal fate. In order to favour this process in ES-derived NS cells, we tested different components of the in vitro environment. For this reason, the LC1-EGFP NS cell line (Conti et al., 2005), was plated onto different substrate-coated dishes (uncoated, poly-ornithine (PO), laminin, fibronectin, PO-laminin, Matrigel; not shown) and treated with different media and supplements at various concentrations either alone or in combination, concurrently or sequentially. We decided to start by sequentially modifying a single parameter with respect to the conditions described in the published protocol (Conti et al., 2005). For our analysis, we focussed our attention on parameters such as morphology, survival and antigenic properties. Generally, initial neuronal differentiation can be induced by growth factors deprivation. However, sudden FGF-2 and EGF withdrawal resulted in rapid death of most of the cells in culture (not shown). Maintenance of EGF was not applicable since it sustains the continuous growth of NS cells (not shown). We also found that complete FGF-2 withdrawal by itself was detrimental to the culture, resulting in gradual degeneration (not shown). We found as best option the complete withdrawal of EGF (as also experienced in our previously published protocol) and a reduction of FGF-2 concentration from 20 ng/ml to 10 ng/ml. As supplements, we maintained the N2 mix but we also included B27 supplement that was important to preserve cell viability and to induce the acquisition of an immature neuronal-like morphology.

In these experiments, different commercial media were also tested (NeuroBasal, DMEM-F12, Euromed-N, RHB-A; Fig. S1). We found that exposure to NeuroBasal medium did not elicit positive effects, resulting in an abrupt morphological change, with cells exhibiting a flat morphology and intracellular vacuoles. Similar effects were observed with DMEM-F12, although the cells exhibited a less flat morphology. Mixing NeuroBasal with DMEM-F12 induced intermediate effects. In our hands, the best results were obtained with Euromed-N medium which did not interfere with the overall survival of the cultures while inducing the cells to acquire a pronounced immature neuronal-like appearance. Because of these initial positive effects, we decided to focus our attention on the latter two media. By combining all of these parameters, we defined the optimal conditions for the neuronal commitment step consisting of exposing the cells to RHB-A or Euromed-N medium, containing 10 ng/ml FGF-2 and N2/B27 supplements (hereafter renamed as “D1 medium”) and at a plating density of 6 × 104 cells/cm2 on uncoated tissue culture treated plastic. Three day exposure of NS cells to these conditions had remarkable effects in terms of survival and attachment of the cells with consequent progressive morphological maturation of the cells (Fig. 1, phase contrast). We considered this pre-differentiation/
neuronal commitment step as the key determinant in the obtainment of a healthy culture enriched in terminal neurons. In order to determine the gene expression and antigenic profile of NS cells treated for three days with D1 medium, we collected samples processed them both for microarray analysis and western blot/immunofluorescence with various antibodies. Affymetrix oligonucleotide microarrays (GeneChip 2.0 array platform) was employed to detect the mRNA expression status of over 34,000 mouse genes in D1 treated versus self-renewing NS cells. Of the 34,000 genes queried, a total of 3038 genes were upregulated, whereas 3411 genes were downregulated. Many genes were represented several times in the chip and showed concordant expression, thereby validating the hybridization. The majority of the characterized genes regulated were involved in the regulation of cellular metabolism (37.88%) and of physiological process (16.59%) (Fig. S2). The expression of molecules involved in transport (12.43%) and cell organization and biogenesis (12.09%) were also modiﬁed (Fig. S2). In addition, D1 treated NS cells exhibited a wide modulation of cell cycle genes (6.54%), including cyclin B2, D1, and p21, whose regulation has been implicated in the initial differentiation of neuronal progenitors (Fig. S3). The three days D1 treatment also resulted in the downregulation of Nestin and the upregulation of neuronal genes (Table 1).

**Table 1**

<table>
<thead>
<tr>
<th>Protein Function</th>
<th>Expression ratio</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nestin</td>
<td>Cytoskeleton</td>
<td>Down-regulated 8.08 fold</td>
</tr>
<tr>
<td>Tenascin C</td>
<td>Cell adhesion</td>
<td>Down-regulated 4.16 fold</td>
</tr>
<tr>
<td>Sox21</td>
<td>Transcription</td>
<td>Up-regulated 2.47 fold</td>
</tr>
<tr>
<td>N-CAM</td>
<td>Cell adhesion</td>
<td>Up-regulated 3.47 fold</td>
</tr>
<tr>
<td>Doublecortin</td>
<td>Neuro migration</td>
<td>Up-regulated 4.25 fold</td>
</tr>
<tr>
<td>Tau</td>
<td>Cytoskeleton</td>
<td>Up-regulated 2.46 fold</td>
</tr>
<tr>
<td>Beta3-Tubulin</td>
<td>Cytoskeleton</td>
<td>Up-regulated 1.04 fold</td>
</tr>
<tr>
<td>NF-M</td>
<td>Cytoskeleton</td>
<td>Up-regulated 1.51 fold</td>
</tr>
<tr>
<td>Synaptogaptin</td>
<td>IV Synaptic compartment</td>
<td>Up-regulated 2.27 fold</td>
</tr>
<tr>
<td>Synaptosomal associated protein 25 (SNAP25)</td>
<td>Synaptic compartment</td>
<td>Up-regulated 1.59 fold</td>
</tr>
<tr>
<td>Synaptosomal associated protein 91 (SNAP91)</td>
<td>Synaptic compartment</td>
<td>Up-regulated 2.06 fold</td>
</tr>
<tr>
<td>Glutamic acid decarboxylase 1</td>
<td>Neurotransmitter synthesis</td>
<td>Up-regulated 6.17 fold</td>
</tr>
<tr>
<td>Glutamate receptor, ionotropic, AMPA2 (α2)</td>
<td>Neurotransmitter receptor</td>
<td>Up-regulated 2.33 fold</td>
</tr>
<tr>
<td>Glutamate receptor, ionotropic, delta 2</td>
<td>Neurotransmitter receptor</td>
<td>Up-regulated 3.25 fold</td>
</tr>
</tbody>
</table>

Summary of 14 different neural and neuronal genes highly modulated in D1 treated LC1-EGFP cells compared to self-renewing LC1-EGFP cell cultures. Genes are grouped according to gene functions. (*) identified with multiple probes.)
Confirmitory results were obtained by immunofluorescence and western blot analyses (Fig. 1). After 3 days in D1 medium, the majority of cells were post-mitotic, as indicated by a reduction in the number of cells immunoreactive for phospho-Histone H3 marker (0.3±0.2%), had lost their neural stem cell identity, as shown by lack of expression of Nestin (observed in only 18.6±3.7% of the cells) (Figs. 1A and B). Expression of the radial glia RC2 marker was also reduced (not shown) while pan6 and Blbp immunoreactivities were still present (Figs. 1A and B), in agreement with the microarray data. Olig2 expression was still sustained in the D1 treated cultures (Fig. 1A), possible due to the presence of FGF-2 in the D1 medium. Finally, both microarray, immunofluorescence and Western blot data indicated up-regulation of genes involved in early neuronal specification, such as Sox2 (Sandberg et al., 2005), N-CAM (Mayer-Proschel et al., 1997; Schmandt et al., 2005), Dcx (Francis et al., 1999; Gleeson et al., 1999) and the immature neuronal marker β3-tubulin (Table 1; Figs. 1A and B). A significant fraction of the cells in culture is negative for mature neuronal markers NeuN (Fig. 1A), TAU (not shown) and MAP2ab (not shown), while exhibited β3-tubulin (70.9±1.1%) or Dcx (17.8±2.3%) immunoreactivity, identifying them as immature neuronal cells or neuroblasts (Fig. 1). Thus, transcription of some neuronal genes seems activated in following exposure to D1 medium conditions (TAU, NeuN) but their translation is still absent. This can also explain the induction of other mature neuronal transcripts (Fig. 1; Table 1) that are not detectable at the protein level at this stage. Finally, only 0.9±0.6% of the total cell population became GFAP-immunoreactive astrocytes (Fig. 1A), indicating that these in vitro conditions efficiently bias ES-derived NS cells toward the neuronal lineage.

Set-up of optimal conditions for the robust in vitro neuronal maturation of ES-derived NS cells

We subsequently set out to define the conditions allowing the complete functional and neurochemical maturation of the cells that could assure rigorous quantitative and qualitative criteria, including robustness and reproducibility. We found that if cultures were maintained for longer periods of time (>7 days) in D1 medium or shifted to other media (Neurobasal, DMEM-F12 or mix of different media) they were losing viability and efficient neuronal differentiation. Because of this, we attempted at gently dissociating the D1 treated cells in order to replate them in different conditions. Following detachment, cells were replated on a number of substrate-coated tissue culture plastic or glass coverslips (uncoated, poly-D-lysine, laminin, fibronectin, PDL-laminin, Matrigel; Fig. S4) and exposed to different media and supplements in vitro at various concentrations either alone or in combination, in concert or sequentially using an approach similar to the one described above for the neuronal commitment process.

We identified as best condition, the plating on laminin-coated surface (Fig. S4) in FGF-2 and BDNF containing medium A, which is replaced after 3 days by B medium for terminal differentiation (see “Materials and methods” for details; Fig. 2A). The B medium was changed every 3 days and contains a reduced dose of FGF-2 (5 ng/mL) and increased amount of BDNF (30 ng/mL). We maintained a minimum dose of FGF-2 in the cultures since it is essential for cell viability. Also, the presence of BDNF allowed to increase the number of surviving cells and exerted a positive effect on the complete neuronal maturation of the cultures, both antigenically and functionally.

When exposed to these conditions, ES-derived NS cells gradually adhered and progressively developed a neuronal morphology as indicated by the appearance of neurites outgrowth, the establishment of complex branched morphology and cell-cell contacts (Fig. 2B). In the differentiating cultures a small number (below 5%) of flat cells, with an astrocyte-like appearance are present and sporadic oligodendrocytes can be identified morphologically.

Importantly, a comparison of cells differentiated in these optimized conditions with respect to the previously published protocol shows a clear increase in cell viability at the late time points (14 and 21 DIV; Figs. 2B–C). Indeed, the original protocol allowed a poor survival after 14 and 21 DIV (34.2±3.7% and 19.5±6.0% for 14 and 21 DIV respectively when compared to 7 DIV; p<0.001 by t-test) while in the new conditions cell viability is maintained at significantly higher levels (90.8±6.8% and 85.8±7.3% for 14 and 21 DIV respectively when compared to 7 DIV; p<0.001 by t-test). A direct comparison of the basic differences between the original protocol and the current procedure is detailed in Supplementary Table 3.

The fact that the viability of the cultures is well preserved and the proliferation is abolished after the three days in D1 conditions indicates that almost the entire population is undertaking a phenotypic conversion from proliferation to differentiation, thus excluding the occurrence of a selection process in a fraction of the cells in culture.

LC1-EGFP cells exposed to the optimized neuronal differentiation conditions efficiently and robustly generate GABAergic neurons

Aiming at thoroughly defining the differentiation efficiency of ES-derived NS cells, we set out to qualitatively analyse the cultures by immunocytochemistry at three different stages of maturation (7, 14 and 21 DIV) followed by quantitative evaluation of the antigenic properties of the stage-specific populations. Analysis of Nestin immunoreactivity in the cultures indicated a time-dependant gradual decrease in the number of cells expressing this immature marker (Fig. 3), being already significantly reduced after 7 DIV (self-renewal conditions: 97.2±2.7%; 7 DIV: 5.8±4.3%) and further decreasing to 3.4±1.0% and 4.3±2.5% at 14 and 21 DIV, respectively.

Similar trends of expression have been observed for other immature markers of NS cells such as RC2 and sox2 which were absent in 21 DIV old cultures (not shown). These results reveal a progressive decline of the developmental potential of differentiating NS cell cultures. Consistently, we found that almost all of the cells are already postmitotic after 7 DIV, as indicated by the small fraction of cells (0.5±0.3%) that stain positive for phospho-Histone H3 that marks cells in M phase. At 14 and 21 DIV no phospho-Histone H3 immunoreactive cells are found (data not shown).

Fig. 3 shows that the majority of the cells have acquired immunoreactive signals for neuronal markers already after 7 DIV (percentage of immunoreactive cells: 51.6±2.6% β3-tubulin, 44.7±3.9% MAP2ab). Noteworthy, at later time points, the number of cells immunoreactive for these neuronal markers further increases (percentage of immunoreactive cells after 14 DIV: 75.0±5.5% β3-tubulin, 64.5±4.2% MAP2ab; percentage of immunoreactive cells after 21 DIV: 84.6±5.3% β3-tubulin, 76.6±2.9% MAP2ab). Note-worthy, at these later time points the immunoreactive signal becomes more intense and the cells show a more complex and branched neuronal morphology (Fig. 3).

The number of cells with a flat morphology and immunoreactive for the GFAP is extremely low in the cultures after 7 DIV (1.8±0.5%, Fig. 3) and did not change after 14 and 21 DIV respectively (Fig. 3). Similarly, O4 immunoreactive oligodendrocytes were only sparely present (not shown). Collectively, these data indicate that these in vitro conditions do not favour the conversion of ES-derived NS cells into the astroglial and oligodendroglial lineages.

We next analysed the expression of specific neurotransmitters in order to define the neuronal subtype composition of the cultures. We found that, after 7 DIV, only a minor fraction of the cells in culture exhibited a detectable immunoreactivity for GABA (Fig. 3) and GAD67 (Fig. S5) while after 14 and 21 DIV, both the intensity of the staining and the percentages of GABA immunoreactive cells raised to a considerable higher fraction (75.1±6.1% and 82.6±6.4% after 14 and 21 DIV respectively).
Fig. 2. Morphological properties of ES-derived LC1-EGFP cells in proliferation and after exposure to neuronal maturation conditions. (A) Schematic representation of the differentiation procedure (see "Materials and methods" for further details). (B) Morphological properties of LC1-EGFP cells under self-renewing conditions and after exposure to the previously developed differentiation protocol and to the new optimized procedure. In self-renewing conditions, LC1-EGFP cells maintained their typical bipolar and lattice-like morphology. When induced to differentiate, LC1-EGFP cells developed cell–cell contacts and spines, the number increasing over time, from 7 DIV to 14 DIV and 21 DIV. At the latter time points, it is evident by the drastic increase of the quota of surviving cells with the new optimized protocol (new protocol) in comparison to the previous protocol published in Conti et al. (original protocol; Conti et al., 2005). (C) Quantitative comparison of cells differentiated in optimized conditions (light gray bars) with respect to the previously published protocol (dark gray bars). This analysis shows a clear augment in cell viability at the late time points (14 and 21 DIV). The percentage of cells is referred to the 7 DIV (considered as 100%).
Taken together, these results suggest that our new protocol allows robust survival of lineage committed ES-derived NS cells which then undergo differentiation into GABAergic neuronal subtype. In these conditions, all the other neuronal subtypes (cholinergic, glutamatergic, dopaminergic and serotoninergic) investigated by immunocytochemistry and RT-PCR are not represented in the cultures at any time-point analysed (not shown). Whether variations of this protocol might give rise to different neuronal subtypes will be questions for future investigation.

Long-term expanded ES-derived NS cells efficiently differentiate into neurons

One of the most stringent criteria that gives value to a neuronal differentiation protocol, in addition to its efficiency in neuronal production, and to a stem cell system is its robustness and reproducibility throughout extensive culturing.

To test whether our differentiation procedure could match this criterion and generate the same proportion of progeny at different passages, we performed seven independent differentiation experiments on cells at different passages, the oldest tested being passage 130, and analysed the compositions of the cultures at different time points by immunocytochemistry. For this analysis we focussed on β3-tubulin, MAP2ab, GABA, and GFAP markers (Table 2). We found that the proportion of neuronal and glial cells is maintained with no significant variations over passages and up to passage 130 (Table 2). Fig. S6 shows an example of this analysis performed on LC1-EGFP cells at passage 99 (Fig. S6).

On the whole, here we have shown that our cell culture conditions allow homogenously growing ES-derived NS cells to acquire antigenic properties of GABAergic neurons and that the neuronal differentiation conditions established are extremely consistent between experiments performed on cells at comparable number of passages and reproducible using cells at different passages (Table 2).

NS cells derived from different embryonic stem cell lines differentiate into neurons

An important concern when considering a neuronal differentiation protocol is whether it is applicable to a variety of cell lines resulting in comparable results. Here we tested our optimized neuronal differentiating conditions on different ES-derived NS cell lines obtained from independent ES cell lines. Three NS cell lines, NS-LC1, NS-CGR8 and NS-R1, derived from 46C, CGR8 and R1 ES cell lines, respectively, were exposed to the new differentiation conditions and their
morphological changes and antigenic properties assayed at different time points.

Fig. 4A shows that the morphology of the differentiating cultures gradually evolves giving rise to cells with neuronal and astrocytic characters. Indeed, the majority of the cells in the cultures progressively develop neurites outgrowth and exhibited a branched morphology, with a minority of the cells showing a flattened appearance. Additionally, some spare cells with an oligodendrocyte-like shape can occasionally occur in the cultures.

We next investigated the antigenic properties of these cultures at different time points by means of immunocytochemistry analysis. Fig. 4B shows pictures of the NS-R1 cultures analysed for Nestin, β3-tubulin, GFAP and MAP2ab immunoreactivities at the different time points considered. In particular, after 7 DIV, only 2.2 ± 0.8% of NS-R1 are Nestin immunoreactive, a percentage which is grossly maintained at later time points (Fig. 4B). On the contrary, neuronal markers such as β3-tubulin and MAP2ab are progressively more present in the culture. A lower percentage of GFAP-immunoreactive astrocytes can be identified as well (Fig. 4B). We also found that the neuronal cells exclusively acquired GABAergic antigenic properties, being immunoreactive for GABA (Fig. 4B) and GAD67 (not shown). The percentage of GABA immunoreactive cells increases and the signal becomes stronger during the differentiation. Overall, the other two cell lines considered, NS-CGR8 and LC1 cells, showed a comparable qualitative and quantitative behaviour in these conditions (not shown), these results being similar to those obtained with the LC1-EGFP cells.

Differentiating ES-derived NS cells develop voltage gated Na+ currents

In order to assess the degree of functional neuronal maturation of ES-derived NS cells exposed to these differentiating conditions, the whole-cell patch-clamp technique was used to monitor the extent of
in vitro differentiation of LC1-EGFP cells into electrophysiologically mature neurons. Importantly, we found a close correlation between cell capability to elicit regenerative voltage responses and the appearance of voltage-gated Na⁺ currents. Figs. 5A–C (lower traces) shows sample voltage-clamp records of the total inward and outward voltage-gated currents elicited at the test potential of −10 mV from a holding voltage of −75 mV, and obtained from three individual LC1-EGFP cells exposed to differentiating conditions for at least 6 DIV. It is evident how the amplitude of the inward Na⁺ current displayed substantial variability from cell to cell.

Significantly, upon switching from voltage- to current-clamping, and during stimulation with rectangular current pulses, the same cell generated quite different voltage responses that were strictly correlated to the amplitude of the Na⁺ currents (Figs. 5A–C, upper tracings). For instance, only the cell displaying a relatively large Na⁺ current (>0.5 nA) showed an action potential with overshooting (Fig. 5C, upper trace), whereas the one with a very small Na⁺ current (<0.1 nA) displayed no regenerative response at all (Fig. 5A, upper trace).

From LC1-EGFP cells exposed to differentiating conditions for less than 6 DIV, the voltage-gated Na⁺ currents were negligible and no regenerative voltage response could be detected (n = 5, not shown). By contrast, the number of cells displaying regenerative voltage responses increased abruptly in cells maintained in the differentiative medium for 6 DIV or longer time, as illustrated in Figs. 5D–E, where the percentage of cells eliciting overshooting and abortive action
potentials (AP) or no AP at all as a function of the number of DIV is shown. In particular, the percentage of cells with overshooting AP increased to 50% and 54% in cells maintained in the differentiative medium between 6 and 14 days (Fig. 5D). As shown in Fig. 5E, this percentage remained almost unchanged during cell maintenance in the differentiative medium for longer time (54% between 15 and 30 days).

Discussion

Expandable sources of neural precursors have several important uses, offering a tool to dissect the mechanisms of neuronal maturation and opportunities for screening novel CNS pharmaceuticals in relevant cell-based assay systems. Presently, however, such applications are limited by the reduced competence to reliably generate large numbers of well characterized antigenically and physiologically mature neurons. The establishment of an in vitro cell culture system consisting of highly neurogenic neural stem cells thus represents one of the key requirements in the field.

Here we demonstrate that exposure of ES-derived NS cells to in vitro fully defined differentiation conditions efficiently drives the cells toward a neuronal lineage and yields functionally mature neurons that are unique in their quality and number. The establishment of an in vitro cell culture system consisting of highly neurogenic neural stem cells thus represents one of the key requirements in the field.

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Here we demonstrate that exposure of ES-derived NS cells to in vitro fully defined differentiation conditions efficiently drives the cells toward a neuronal lineage and yields functionally mature neurons that are unique in their quality and number. In particular, we show that even after long-term in vitro expansion (for more than 130 passages), ES-derived NS cells maintain their capacity to generate a high percentage of MAP2+ve neurons with a GABAergic phenotype and firing action potentials.

With respect to our previously reported studies for NS cell neuronal differentiation (Conti et al., 2005), the major advances here obtained have entailed both survival and the neuronal differentiation efficiency. The improvement has been in fact drastic with respect to the previous protocols that led to a 35–50% of β3-tubulin positive cells after 10–14 days of differentiation and a survival rate of approximately 10–20% after two weeks in vitro. Also, the appearance of electrophysiologically mature neurons was achieved in a small proportion of the cells and only after 4 weeks of exposure to neuronal differentiation protocol. The new differentiative procedure presented here has led to a survival rate of about 90% after 14 DIV and 85% after 21 DIV and allows now 80–85% of the cells in culture to express neuronal markers such as β3-tubulin, MAP2ab and NeuN at this later time point.

The novel neuronal differentiation protocol consists of the sequential exposure of NS cells to fully-defined serum-free neuron-supportive conditions. A distinctive characteristic of our new protocol is the presence of two separate steps, an initial priming procedure that promotes neuronal induction and a second phase that mainly allows neuronal maturation. The neuronal induction is achieved by a 3 days exposure to an EGF deprived medium containing B27 supplement. These conditions have a strong effect on cell cycle exit as demonstrated by the reduced number of cells being immunoreactive for phospho-Histone H3 and by the sharp regulation of cell-cycle-associated molecules. Importantly, this priming procedure provokes the cultures to up-regulate early neuronal markers and restrains the astro-glial differentiation.

Another group has previously described an in vitro priming method applicable to fetal human neural stem cells (Wu et al., 2002). The authors exposed the cultures for 7 days to a cocktail of FGF-2, heparin and laminin and they showed that this priming procedure resulted in an enriched population of neurons in vitro (about 45%) and after transplantation into non-neurogenic areas of adult rat intact CNS (Wu et al., 2002). Although some points of contact can be found with respect to the present study, our priming procedure differs on various key aspects. Indeed, Wu et al. used cells of human origin routinely grown as neurospheres. Exposure of these cultures for 7 days to their
priming procedure, differently from us, did not induce cell cycle exit and a decrease of progenitors markers expression, being most if not all their cells in culture still Nestin positive at this time. On the contrary, our procedure not only induced a rapid and marked decrease of neural markers like Nestin and Tenascin C after only three days but also led to a parallel appearance of early immature neuronal markers.

Generally, in vitro studies designed to assess the neuronal differentiation potential of ex vivo expanded NSCs have been performed mostly on neurospheres at early passages (passages 2–10). However, neurospheres have considerable restrictions as a stem cell propagation system that might lessen the ability to appreciably expand these cells in vitro without loss of their neurogenic potential. Among the general protocols for in vitro neurospheres differentiation, the most efficient consists of sphere attachment on poly-ornithine or laminin treated substrate followed by mitogens removal (Vescovi et al., 1993). In these conditions, though, a limited amount (less than 15%) of MAP2+ve neurons was found (Gritti et al., 1999). Other protocols relied on partial EGf withdrawal, and in these conditions, fetal-derived mouse neurospheres generated roughly 20% MAP2+ve neurons (Grandbarbe et al., 2003).

Differently from neurospheres, NS cells represent a nearly pure adherent neural progenital glia-like stem cell population in vitro. Particularly, the distinguishing advancement of the NS cell system is its reduced complexity. Interestingly, their homogeneity and growth within a monolayer might provide the critical conditions for the exceptional neurogenic potential observed as yet reported for adult mouse SVZ-derived NS cells (Goffredo et al., 2008; Pollard and Conti, 2007) and now for ES-derived NS cells. The main difference between ES-derived and adult SVZ-derived mouse NS cells, beside the percentage of neurons obtained after 3 weeks of exposure to differentiation protocols (85% and 65%, respectively), consists in the reduced number of astrocytes generated. Indeed, in differentiated ES-derived NS cell cultures only 2–5% of the cells acquire an astroglial identity with respect to the 35% observed in the differentiated adult mouse SVZ NS cells. This difference may underline intrinsic neurogenic differences between the NS cell lines from different sources and not due to the diverse protocols employed. In fact, exposure of adult mouse SVZ-derived NS cells to the conditions here described for the ES-derived NS cells does not result in significative differences on the previously reported percentages of neurons and astrocytes (not shown; Goffredo et al., 2008).

Importantly, neurons generated from ES-derived NS express a set of markers specific to GABAergic neurons, a commonality that these cells share with adult SVZ-derived NS cells (Goffredo et al., 2008a, b). This might be in agreement with earlier studies that have reported the in vitro differentiation of neural precursor cells into GABAergic phenotypes and suggested that the GABAergic phenotype is the default differentiation pathway for expanded neuronal precursor cells. In our conditions, after 21 days of differentiation about 80% of the neurons in culture are GABAergic as indicated by the GABA immunoreactivity with a major portion (90%) of this GABAergic population also strongly immunoreactive for GAD67.

Beside the neuronal antigenic maturation of the differentiating ES-derived NS cell cultures, an important consequence of the enhanced differentiation is the improved functional maturation of NS-derived neurons. Overall, our electrophysiological data clearly indicate that NS-ES cells could be efficiently transformed into functionally mature neurons. Indeed, the ability of NS-ES cells to generate overshooting action potentials increased considerably and rapidly following few days of maintenance in the neuronal differentiative medium, this capacity being preserved at late time points.

Significantly, co-culturing with primary astroglial cells or drug exposure is not necessary to achieve high neurogenic capacity and functional neuronal maturation as shown for adult hippocampus derived neural progenitors (Hsieh et al., 2004; Palmer et al., 1997; Song et al., 2002). Our differentiation protocol is achieved by applying different cell culture media and does not require lineage selection of postmitotic neuroblasts. In addition, it is worth noting that under these defined conditions, the generation of neurons is extremely robust and reproducible across passages in vitro (Table 2) and different cell lines, a feature that may results useful for molecular, biological, and screening applications.

Conclusion

In conclusion, our neuronal differentiating conditions enable ES-derived NS cells to acquire antigenic and electrophysiological properties typical of mature neurons. The homogeneity and stability of NS cell system enabled us to systematically and reproducibly achieve progressive, efficient, robust, and reliable conversion of ES-derived NS cell populations into neurochemically mature and electrophysiologically active GABAergic neurons.

Because of these properties, we believe that ES-derived NS cells and their mature derivatives offer a unique system for genetic and chemical screens. Additionally, given that ES-derived NS cells can be readily and efficiently induced to undergo GABAergic differentiation, these cells may provide an useful tool for studies of neuronal differentiation and in models of neurodegenerative diseases, e.g. Alzheimer’s disease (Rissman et al., 2007) and Huntington’s disease (Zuccato and Cattaneo, 2007), and neurocognitive diseases, e.g. schizophrenia or bipolar disorders resulting from an abnormal activity of the corticolimbic circuitry (Benes and Berretta, 2001).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2009.02.007.

References


