Quantitation and functional characterization of neural cells derived from ES cells using nestin enhancer-mediated targeting in vitro

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Summary
To gain insight into early events of neurogenesis, transgenic embryonic stem (ES) cells were generated using the enhanced green fluorescence protein (EGFP) reporter gene under the regulatory control of the neural stem cell marker, nestin. The expression of EGFP in undifferentiated ES cells suggested that the onset of endogenous nestin occurred before neurulation. Upon differentiation of ES cells, the EGFP expression became confined to the neural lineage and asynchrony in ES-cell-derived neural differentiation was evident. The EGFP intensity was prominent in the proliferative progenitors and unipolar neurons, whereas downregulation occurred in differentiating bi- and multipolar neurons. This was corroborated quantitatively using flow cytometry where maximal generation of neural progenitors was observed 4–12 days post-plating. The proliferative potential of neural progenitors as well as glia, in contrast to post-mitotic neurons, was also evident by time-lapse microscopy. The functional characterization of progenitors revealed an absence of voltage-activated inward currents, whereas the Na\(^+\) current (I\(_{Na}\)) was detected prior to Ca\(^{2+}\) currents (I\(_{Ca}\)) in differentiating neurons. Additionally, inhibitory receptor-operated channels could be detected at these early stages of development in bi- and multipolar neurons suggesting that the pre-committed progenitors had retained their intrinsic ability to give rise to functional neurons. This study sheds new light on early events of neurogenesis defining the quantitative and qualitative aspects and demarcating the functional neural cell types from ES cells in vitro.

Movies available on-line

Key words: ES cells, Nestin, EGFP, Neural progenitor, Neurogenesis

Introduction
Neurogenesis is considered to be the most complex event of organogenesis during embryonic development involving a precise signalling and cellular interaction cascade in order to generate a functional neural network. This comprises various subtypes of neurons, astroglia and oligodendroglia. Unlike the earlier belief that mammalian nervous system stem cells are confined to the embryo and that adult brain lacks the capacity to regenerate, recent studies demonstrate the presence of neural stem cells in both fetal and adult hypothalamus, olfactory bulb, subventricular zone and the dentate gyrus of hippocampus (Reynolds and Weiss, 1992; Gage, 2000; Bjornson et al., 1999; Doetsch et al., 1999; van der Kooy and Weiss, 2000). Since there is generally a recurrence of embryonic phenotypes in adults after injury (Laywell et al., 1992; Lendahl, 1997; Rossi et al., 1997), whether as a means of repair or merely a default state, it is mandatory to investigate the early embryonic events in order to understand the significance of this phenomenon. Hence, pluripotent embryonic stem (ES) cells recapitulating the in vivo events in a relatively precise manner may serve as an ideal model system for the investigation of early embryonic developmental processes (Okabe et al., 1996). In particular, tissue-specific promoter-mediated targeting is an efficient approach in the identification, isolation and functional characterization of lineage-specific populations in the heterogeneous cell mass of the ES-cell system (Kolossov et al., 1998). In the present study the ES cells have been targeted with neural-specific enhancer driving live reporter expression in order to understand early neurulation events.

The intermediate filament protein, nestin, a well known marker for neural stem cells, is expressed in the majority of mitotically active CNS and PNS progenitors (Lendahl et al., 1990; Lendahl, 1997; Cattaneo and McKay, 1990; Mujtaba et al., 1998); it is downregulated upon differentiation (Zimmerman et al., 1994; Lothian and Lendahl, 1997) and reported to reappear upon injury (Lendahl, 1997; Krum and Rosenstein, 1999; Namiki and Tator, 1999; Pekny et al., 1999). Thus, the cells expressing nestin show all the characteristic features of stem cells such as multipotency, self-renewal and regeneration. Hence, nestin could serve as an efficient candidate marker gene in order to unravel early neurogenic proceedings from ES cells in vitro. Unlike Tα1 tubulin, the unipotent neuronal progenitor marker whose expression is confined only to the pre- and post-mitotic neurons (Wang et al., 1998; Roy et al., 2000a; Roy et al., 2000b), nestin represents a more broad spectral, multipotent neural lineage marker expressing not only in neurons but in glia as well (Hockfield and McKay, 1985; Messam et al., 2000). Thus, the
generation and demarcation of both neurons and glia would pave the way in exploring the underlying mechanism(s) of neurogenesis as well as gliogenesis in the ES-cell model system.

Previous studies on the nestin gene using transgenic mice demonstrated that the second intron has the necessary cis-acting enhancer motifs for driving the reporter gene expression in a neuron-specific manner in the developing CNS (Zimmerman et al., 1994; Lothian and Lendahl, 1997; Josephson et al., 1998; Lothian et al., 1999; Yaworsky and Kappen, 1999). Accordingly, the present investigation was carried out to identify, quantify and functionally characterize the ES-cell-derived, development-dependent, proliferating neuronal progenitors as well as the differentiating neurons based on nestin intron-II-driven EGFP expression. This study provides clues to the multifaceted and dynamic process of neurogenesis using the powerful model of stem cell differentiation in vitro that would otherwise have been a complicated task in vivo.

Materials and Methods

Vectors

The Nes 1852 tk/lacZ, a human nestin promoter vector construct was a kind gift of Urban Lendahl, Sweden (Lothian and Lendahl, 1997). The 2 Kb nestin intron II enhancer-tk restriction fragment was excised from the aforementioned vector construct using HindIII and NotI restriction enzymes. Subsequently, this fragment was subcloned into the pEGFP1 vector (Clontech, Germany) at the SalI site by blunt end ligation and termed h-Nestin-EGFP, where the EGFP reporter is under the regulation of the nestin-tk promoter. The insertion and orientation of the promoter to pEGFP vector was verified by both restriction digestion and sequencing using an automated sequencer (ABI). To obtain the h-Nestin-EGFP vector construct, the nestin intron II was released from h-Nestin-EGFP by SmaI restriction digestion and by subsequent recircularization of the vector.

Cell culture and transfection

The ES cells of the D3 line were maintained as described before (Wobus et al., 1991). Briefly, ES cells were grown on inactivated feeder cells in DMEM supplemented with 15% fetal bovine serum (FBS), non-essential amino acids, 2 mM glutamine, 50 µg/ml Pen- Strep (all from Gibco BRL, Germany), 0.1 mM β-mercaptoethanol (Sigma, Germany), and 1000 U/ml recombinant murine leukemia inhibitory factor (LIF, ESGRO, Gibco). In separate experiments, following linearization using the HindIII restriction enzyme ~5x10⁶ cells were transfected independently by electroporation using ~30 µg of the h-Nestin-EGFP or tkEGFP reporter constructs, respectively, following the standard protocol. The Neo⁵ clones were picked after 10-12 days of G418 selection and propagated using the same medium. G418 selection was maintained throughout the propagation period.

Neuronal differentiation

Differentiation of ES cells was initiated by cell aggregation following the hanging drop method (Wobus et al., 1991) in DMEM supplemented with 10% FBS. All-trans-retinoic acid (RA) was used as the inducing agent for neuronal differentiation as described (Strubing et al., 1995) with slight modifications. The neural differentiation was also monitored using the conditioned medium (Okabe et al., 1996). Since the time window (~7-10 days post-plating) for optimal neural progenitor generation was similar irrespective of the medium used, the data from RA-induced progenitors are presented here for convenience. In brief, following trypsinization, the ES cells (500cells/20µl drop) were exposed to RA (10⁻⁷ M) for 3 days during hanging drop followed by suspension culture of embryoid bodies (EBs) for 4 days and plating without RA. Alternatively, RA was added to the medium while plating the EBs and the medium was replenished with fresh medium without RA on the fourth day post-plating (i.e. d7+4).

RT-PCR

Total RNA was isolated from the undifferentiated EGFP-positive transgenic nestin ES-cell clones and the wild-type ES-cell line D3, grown with or without feeders, as well as from murine blastocysts using high pure RNA isolation kit (Roche Molecular Biochemicals, Germany). To ascertain the presence of nestin transcripts in these cells, the total RNA was subjected to reverse-transcription-based PCR following the manufacturer’s instructions (Gibco-BRL). The nestin-specific primers (5’GGATACACCTTATTCAAGG 3’ and 5’CCGCCCTGAAATTACTCT 3’; GenBank accession no. C78523) were designed from the retrieved mouse cDNA sequence, which also corresponded to the C-terminus of the latest reported full length mouse nestin gene (GenBank accession no. AF076623) at positions 5959-5940 and 5481-5500, respectively. The house-keeping HPRT (hypoxanthine phosphoribosyltransferase) (Johansson and Wiles, 1995) and β-actin primers were used as internal positive controls for PCR and designed accordingly to decipher the genuineness of amplified products, based on their size from cDNA, but not from contaminating genomic DNA. The RNA samples without the reverse transcriptase served as the negative control, and the PCR product from each sample was resolved on the agarose gel and observed under a UV-transilluminator.

FACS analysis

The EGFP expression of ES-cell-derived clones was analyzed at various developmental stages using a FACSCalibur™ flow cytometer (Becton Dickinson) equipped with a 488 nm argon-ion-laser (15 mW) as described (Kolossov et al., 1998). In brief, about 10,000-50,000 viable cells were analyzed per sample after isolation using trypsinization (0.1% trypsin and 0.01% EDTA) for 2-5 minutes at 37°C. Subsequently, the cells were resuspended by gentle trituration using PBS containing 1 mM Ca²⁺ and 0.5 mM Mg²⁺. Untransfected D3 line ES cells were used as negative controls. The emitted fluorescence of EGFP was measured in log scale at 530 nm (FITC band pass filter) and analyses were performed using CellQuest® software (Becton Dickinson).

Immunocytochemistry

The specificity of the nestin expression was verified by immunocytochemistry using antibodies against neurons and glia following the standard protocol. In brief, the ES cells and EBs at various stages of development grown on glass coverslips were washed with 0.1 M PBS, pH 7.4 and fixed with 4% paraformaldehyde for 20 minutes. After washing again with PBS the cells were permeabilized with solution containing 0.25% Triton X-100 and 0.5 M ammonium chloride in 0.25 M TBS, pH 7.4 for 10 minutes and blocked with 4% goat serum and 0.8% BSA for 1 hour. Subsequently, the cells were exposed to either of the primary antibodies: anti-nestin (Rat-401, DSHB, University of Iowa), anti-MAP2, anti-synaptophysin or anti-GFAP (all from Sigma chemicals) for 3 hours at room temperature. After washing (0.25 M TBS, three times for 10 minutes each), the cells were treated with Cy3-conjugated secondary antibody for 1 hour at 37°C for fluorescent labelling. Finally the cells were washed three times with TBS and dehydrated with ethanol gradients, followed by xylene treatment and mounting with entellan on glass slides. In each case the negative control was performed with the substitution of respective primary antibodies with goat pre-immune serum. The slides were observed under a fluorescent microscope to detect EGFP as well as Cy3-labeling.
Dissociation of EBs and preparation of single cells

For isolation, 12-16 EBs were dissociated using collagenase B (Roche Molecular Biochemicals, Germany) and re-plated on gelatin (0.1%)-coated glass coverslips as described (Maltsev et al., 1994). In brief, EBs were dissected and rinsed with PBS followed by collagenase B treatment (0.1% in PBS) for 30 minutes at 37°C. Subsequently, collagenase was replaced with a medium containing: 85 mM KCl; 30 mM K2HPO4; 5 mM MgSO4·7H2O; 1 mM EDTA; 5 mM NaATP; 5 mM Na-Pyruvate; 5 mM Creatin; 20 mM taurin and 20 mM glucose; pH 7.2). The cells were stirred slowly for 30 minutes, suspended by gentle trituration in DMEM medium and plated onto gelatin-coated glass coverslips. In initial experiments the central and peripheral regions of the EBs were separated and dissociated individually. Since the pattern of differentiation was similar in these two preparations, whole EBs were used for dissociation. Single isolated cells were used for immunocytochemical characterizations after 2-5 days of re-plating either with parallel cultures or with the same, subsequent to electrophysiological investigations.

Estimation of EGFP intensity and electrophysiology

The semiquantitative estimation of EGFP intensity was performed as described (Kolossov et al., 1998). For the analysis, the EGFP fluorescence intensity comprising the whole area of the cell was integrated and average fluorescence intensities determined in counts.

For patch clamp recordings, isolated neurons of different developmental stages were investigated, using the whole-cell patch clamp technique (Hamill et al., 1981). The cells were voltage-clamped using an EPC-9 amplifier (Heka, Germany), held at –80 mV and depolarizing pulses or ramps were applied (for detail, see figure legends). For the registration of inward currents ramp depolarizations were performed and INa was inhibited by tetrodotoxin (TTX, 0.1 μM).

For estimation of voltage-dependent Ca2+ currents, the extracellular solution was exchanged to a Na+-free solution containing Ba2+ as charge carrier. The expression of the various subtypes of voltage-dependent Ca2+ channels was tested using selective antagonists ([Isradipine, ω-Conotoxin GVIA (ω-CgTX), ω-agatoxin IVA (ω-AgaTX), Almone Labs, Israel), which were bath added. For the recording of receptor-operated currents, substances were applied (15 milliseconds) through a puffer pipette connected to a pressure ejection system (General Valves, USA) placed into the vicinity of the cell of interest (holding potential (HP) –80 mV). The receptor-operated currents were characterized by applying competitive antagonists via the gravitational perfusion system. The ionic nature of these currents was analyzed using subtracted voltage ramps. Data were acquired at a sampling rate of 10 kHz, filtered at 1 kHz, stored on hard disk and analyzed off-line using the Pulse-Fit (Heka) software package. Averaged data are expressed as means±s.e.m. Membrane capacity was determined on-line using the Pulse acquisition software program (Heka). Statistical analysis was performed using paired or unpaired Student’s t-test, and a P value of <0.05 was considered significant.

The glass coverslips containing the cells were placed into a temperature-controlled (37°C) recording chamber and perfused continuously with extracellular solution by gravity at a rate of 1 ml/minute. Substances were applied by exchanging the solution in the chamber, a 90% volume exchange was achieved within approximately 20 seconds. Patch pipettes (2-4 MΩ resistance) from borosilicate glass from Clark (Electromedical Instruments, UK) were pulled using a Zeitz puller (DMZ, Germany). The solutions used had the following composition. Standard external solution, 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM glucose and 10 mM Heps (pH 7.4, adjusted with NaOH). External solution for measurement of INa: 120 mM D(–)-N-methylglucamine, 10.8 mM BaCl2, 5.4 mM CsCl, 1 mM MgCl2, 10 mM glucose and 10 mM Heps (pH 7.4, adjusted with HCl). Normal intracellular solution: 50 mM KCl, 80 mM K-Aspartate, 1 mM MgCl2, 3 mM Mg2ATP, 10 mM EGTA and 10 mM Heps (pH 7.2, adjusted with NaOH). Internal solution for recording of inward currents: 120 mM CsCl, 1 mM MgCl2, 3 mM Mg2ATP, 10 mM Heps and 10 mM EGTA (pH 7.2, adjusted with CsOH). All these chemicals were purchased from Sigma (Germany). The toxins were aliquoted, diluted in normal external solution and frozen at –22°C prior to use.

Online supplemental material (time-lapse microscopy)

For live monitoring of neurogenesis, one day after isolation (d7+7) the cells were continuously observed for 3 days. An inverted microscope (Axiovert 100, Zeiss) equipped with a movable computer controlled stage (Nikon, Germany), an objective 20× (Plan-Neofluar, Zeiss) and a temperature/CO2-controlled chamber (Nikon) were used. Cells were monitored at 30 minute intervals using alternate transmission and fluorescent excitation light with a conventional halogen lamp and a FITC filter set. Images were acquired using a colour video camera (Sony, DVC 950P, AVT Horn, Germany) controlled by the Lucia software (Nikon) and digitized on-line via a video frame grabber card (Matrox Corona, Nikon). For fluorescence pictures, the integration mode of the camera (10 single pictures) and a video-based buffer device (Sony MPU-F100P, AVT Horn) were employed. Movies 1 and 2 depict Fig. 5A and B, respectively, showing the neuro- and gliogenesis from neural progenitors. The cells subsequent to the monitoring were subjected to immunocytochemical verification and neural specification using neuronal and glial specific antibodies (see http://jcs.biologists.org supplemental).

Results

Establishment of stable transgenic nestin ES-cell clones and monitoring of EGFP expression

A number of stable G418 resistant transgenic ES-cell clones of the line D3 were generated by transfection of the h-Nestin-EGFP construct. Several of these selected clones were differentiated into various neural phenotypes following the in vitro differentiation protocol as described before using retinoic acid as the inducer and simultaneously monitored for EGFP expression. 96% of the G418-resistant nestin ES-cell clones (n=100) were observed to be EGFP positive even at the undifferentiated state (Fig. 1A-C). Typically, the clones exhibited a heterogeneous expression pattern of EGFP during propagation on feeder (Fig. 1B,C) or feeder-free cultures indirectly implying the nestin transcription to be active even in undifferentiated ES cells prior to any lineage commitment. Since almost all of our selected, undifferentiated ES-cell clones happened to be EGFP positive, the possibility of non-specificity due to positional effect could be ruled out. Additionally, the non-specific EGFP expression pattern due to the constitutively active heterologous thymidine-kinase (tk) basal promoter was also excluded by performing another set of transfections using the tkEGFP promoter construct without the nestin intron II segment. In less than 15% of these G418-resistant clones only weak EGFP expression was detected (Fig. 1D,E). This is in line with the observation by Lothian and Lendahl who demonstrated the absence of any ectopic lacZ expression driven by the same tk basal promoter in transgenic mice (Lothian and Lendahl, 1997).

Validation of early onset of endogenous nestin and concomitant EGFP expression

To correlate nestin-driven EGFP expression in undifferentiated ES cells with endogenous nestin expression, both, RT-PCR and immunostaining were performed. As shown in Fig. 2, by RT-
PCR with total RNA isolated from undifferentiated ES cells from one of the EGFP-positive transgenic clones (clone-1) as well as from untransfected D3 cells grown in presence or absence of feeders using sequence specific nestin primers, a PCR product of the expected 478 bp size was amplified. Interestingly, the nestin product was detectable in the murine blastocyst RNA (Fig. 2, lower panel, lane 14), but with a comparatively low intensity. This is probably caused by either a low transcript level or low initial RNA content in the RT reaction containing the blastocyst sample because, by using two housekeeping primers (HPRT and β-actin), the respective apparent product intensities with the blastocyst sample were low compared with that of other samples. Hence, we took double the quantity of reverse transcribed first strand from the blastocyst sample in order (1.5 times loading sample volume on gel) to scale up the visible intensity of the product during PCR (Fig. 2, lower panel, lane 8). We further corroborated this finding by immunostaining using a monoclonal antibody against nestin. All the EGFP-positive cells were nestin positive in the feeder-free cultured transgene ES cell clone-1 (Fig. 1G,H). Immunostaining with a monoclonal antibody against nestin in the feeder-free cultured transgenic ES cell clone-1 indicated that all EGFP-positive cells were nestin positive (Fig. 1G,H). In addition, preliminary studies indicate that the inner cell mass (ICM) of murine blastocysts is nestin positive (data not shown), confirming the observation in ES cells. Unlike earlier reports on nestin expression (Okabe et al., 1996; Lee et al., 2000) our findings imply that its onset occurs much earlier than neural plate induction at E7.5.

Nestin intron-II-driven EGFP expression profile during neural differentiation and neurogenic progression

The transition from uncommitted ES cells to a neural lineage-defined state was brought about by RA exposure of EBs. To rule out clonal diversity several (n=6) of the clones were

**Fig. 1.** Human nestin intron-II-driven EGFP expression in undifferentiated ES cells and corresponding immunocytological expression pattern. (A) A single NeoR clone after electroporation of h-Nestin-EGFP construct and G418 selection for 12 days shows heterogeneous EGFP expression. (B,C) The EGFP expression in ES cells (arrow) remains patchy during propagation on mitotically inactive feeders (arrow head) in clone-1. (D,E) Weak EGFP expression in one of the few (<15%) EGFP-positive ES-cell clones transfected with tkEGFP. (B,D) Combined transmission and fluorescent light; (C,E) fluorescence light alone. (G,H) A complete overlap between EGFP expression (G) and (H) nestin-immunoreactivity was observed in undifferentiated ES cells during feeder-free propagation. Bars, 100 μM (A), 75 μM (D,E), 50 μM (B,C,G,H).
Nestin transgenic ES cells and in vitro neurogenesis

analyzed to examine the nestin intron-II-driven EGFP expression profile and specificity in ES-cell-derived neurons during differentiation. During cell aggregation and commencement of differentiation, localized expression of EGFP in EBs marked the transition. Moreover, under no time points studied was there ever complete absence of EGFP-expressing cells in the EBs. After 1 or 2 days post-plating (d7+1/2), the EBs showed a few intense bright shining areas (Fig. 3A,B). By d7+2/3, the cells at the periphery exhibited an epithelial phenotype implicating the differentiation into ectodermal lineage. Starting from d7+4, outgrowths from the central part of the EB with areas of intense EGFP expression were detected (Fig. 3C,D). This further signified the presence of neuroepithelial cells as confirmed by immunostaining with the nestin antibody (Fig. 3J,K).

By d4-6 of plating, few EGFP-positive unibi-/bi-/multipolar neurons were observed at the periphery of the differentiating EBs along with the glial cells, most probably the radial glia (Rat 401 positive) (Hockfield and McKay, 1985). However, the number of neurons with distinct neurite outgrowths significantly increased with time after plating, associated with weakening of EGFP expression (Fig. 3D). By d8-15, bundles of neurons forming extensive networks were observed (Fig. 3E-H). As would be expected, progressive neurogenesis was accompanied by an increase in the number and the length of neurite outgrowths (Fig. 3, compare E with F-H). Most of the terminally differentiated neurons lost EGFP, thus mimicking the endogenous nestin expression pattern as well as reporter gene expression in vivo (Zimmerman et al., 1994; Lothian and Lendahl, 1997). Nevertheless, in many differentiating neurons with distinct neurite outgrowths weak EGFP expression was preserved; this was probably due to accumulation because of its slow metabolic rate (Fig. 3F-H). No significant differences were noticed in the neural differentiation pattern, irrespective of the application of RA, during hanging drop preparation or plating in contradiction to an earlier report (Rohwedel et al., 1999).

Neural specificity of EGFP expression during differentiation

Immunocytochemical studies on whole EBs (Fig. 3J-M) confirmed neural specific confinement of nestin enhancer-driven EGFP expression at different developmental stages (d7+4 to d7+12). A similar neural differentiation pattern was observed in isolated cells (Fig. 4A-K). All EGFP-positive cells stained with the antibody directed against nestin, proving the neural-specific expression of EGFP. These EGFP+/nestin+ cells (Fig. 3J,K; Fig. 4C-D) were thought to be neural progenitors because of their proliferation (BrDU+ or Ki67+; data not shown) (Fig. 5; time-lapse observation) and differentiation potential, giving rise to neurons with uni-, bi- and multipolar morphologies as well as glia; as discerned by immunostaining with both neuron (synaptophysin, MAP2) and glial (GFAP) specific antibodies, respectively (Fig. 3L,M; Fig. 4E-K). The differentiated bi- and multipolar neurons with longer processes were either weak or negative for EGFP (Fig. 4A,B), but stained positively with MAP2, a marker for post-mitotic neurons (Fig. 3L,M; Fig. 4J,K). These cells maintained synaptic connections as evident from synaptophysin staining (Fig. 4G,H) and appeared to form networks with adjacent neurons and glia. More often differentiating neurons grew on top of or adjacent to a

![Figure 2](image2.png)

**Fig. 2.** RT-PCR revealed the presence of nestin transcripts in undifferentiated ES cells as well as murine blastocysts. The upper panel shows nestin transcripts from D3 undifferentiated ES cells (lane 2), transgenic h-Nestin-EGFP ES cells (n-ES cells; lane 5) grown on feeders and from D3 undifferentiated ES cells grown without feeders (lane 8). The lower panel shows nestin transcripts in the transgenic ES cells without feeders (lane 2) and in the murine blastocysts (lanes 8,14). The length of the nestin PCR product corresponds to the expected size of 478 bp. The corresponding products for housekeeping HPRT (upper panel, lanes 4,7,10; lower panel, lanes 5,10) and β-actin (lower panel, lanes 6,12) are shown as controls. Lanes 3,6,9 (upper panel) and 3,4,7,9,11,13 (lower panel) represent the respective negative controls where the reverse transcriptase was omitted from the reaction.
monolayer of flat glial cells establishing extensive connections with each other (Fig. 4J,K). These observations further confirmed that the EGFP/nestin positive neural progenitors retained the ability of multipotentiality since they were able to generate MAP2-positive neurons and GFAP-positive glial cells upon differentiation. Notably, at every developmental stage investigated there was a representative population from proliferating (BrDU+; Ki67+; nestin+) and differentiating cells (MAP2+; GFAP+), indicating an asynchronous profile in neural differentiation.

Monitoring of neural differentiation with time-lapse microscopy

A clearer picture of neurogenic progression emerged in time-lapse experiments. As seen in Fig. 5A, a single EGFP-positive progenitor was observed to divide into two and then into three cells. The triplet underwent positional changes between 6 and 10 hours after the inception of monitoring (see movie; http://jcs.biologists.org/supplemental). Subsequently, one of these cells underwent morphogenic change and showed neurite outgrowth to become uni- and then bipolar. At subsequent time
periods the extension of a neurite in association with migration and interaction with other cells became evident from this observation (Fig. 5A). One of the other two cells exhibited further divisions at 23 hours (data not shown) and 36.5 hours, indicating the probable occurrence of self-renewing asymmetric division and retention of stem/progenitor potential. Many of the bipolar neurons did undergo a further morphogenic change into multipolar ones, depending on their interaction with neighbouring neurons or glia (data not shown), whereas some remained bipolar until the end of monitoring (Fig. 5A). These changes indicate the co-existence of a diverse phenotypic population of mature neurons and neural progenitors at any given time during neuronal differentiation from ES cells in vitro. Similarly, the generation of cells with glial phenotype with subsequent division (Fig. 5B; 55 hours and 58.5 hours), migration and interaction with each other could be clearly depicted from EGFP-positive progenitors (Fig. 5B; 55-66.5 hours). The noteworthy feature was both symmetric and asymmetric division of progenitor and, unlike the neuronal cells, the glial cells underwent further division after undergoing transformation from a flat (Fig. 5B; 54 hours) to a round phenotype (Fig. 5B; 54.5 hours) like the progenitor. However, in both cases weakening of EGFP remained associated with progressive morphogenesis into neuron and glia.

Flow-cytometric quantification of neurogenesis from ES cells in vitro
The qualitative aspect of neurogenesis in vitro was complemented by a quantitative assessment performing flow-
cytometry at different stages of development. In several of the EGFP-positive clones analyzed (n=4), the undifferentiated ES cells exhibited about 40-50% of bright and weak EGFP fluorescence each when compared with D3 wild-type ES cells (Fig. 6A). Upon differentiation by cell aggregation, RA treatment and prior to plating (Fig. 6A; left panel, 7+0), there was a clear leftward shift in the EGFP peak (28% weak: 20-100 intensity range; and 2% bright: 100-1000 range) with the majority (70%) of cells being EGFP negative (1-20 range). In the post-plating EBs, EGFP fluorescence further intensified in line with the microscopic observation pattern reported above. About 1-6% of the total bright shining population of cells from the RA-treated group (clone-1) was very intense after plating, with EGFP intensity levels beyond the 1000 range (Fig. 6A).

This population was observed even up to 24 days after plating. As seen in Fig. 6A, there was a rightward shift in the EGFP-positive peak in the 4 day post-plated EBs (70% EGFP⁺⁺⁺: 20-10,000 range; 30% EGFP⁺⁺: 1-20 range) indicating a biphasic distribution pattern that remained almost the same in the 10-12 day post-plated EBs (52-57% EGFP⁺⁺⁺ and 43-48% EGFP⁺⁺) and gradually decreased with the course of time from 12 to 24 days post-plating (20% EGFP⁺⁺⁺ and 80% EGFP⁺⁺). This clearly complied with an increase in the number of post-mitotic differentiating neurons during the course of differentiation as directly monitored with time-lapse microscopy. The time course of the neurogenic development indicated that the percentage of the brightest EGFP-positive (EGFP⁺⁺⁺: 1000-10,000 intensity range) progenitor population in the plated EBs remained stable between d7+4 (7.2±2.0%) and d7+10/12 (6.0±1.3%) of plating and declined to 2.5±1.0% after 24 days of plating (Fig. 6B). Similarly, the proportion of cells belonging to the bright EGFP-positive (EGFP⁺⁺⁺: 100-1000 range) group showed gradual decline in EGFP intensity (from 27.9±3.3% to 10.9±5.0%), implying the loss of residual EGFP in these differentiating neural populations during a longer plating period. By contrast, the proportion of weak EGFP-positive cells (EGFP⁺⁺: 20-100 range) remained unchanged (24±11.3% to 28.7±2.3%) and an almost exponential rise in the EGFP-negative cells from 36.1±2.0% to 62.6±17.3% could be observed between day 4 and day 24 after plating (Fig. 6B). Taken together, these data suggested an asynchronous neural differentiation profile and
that the maximum number of neural progenitors was generated from ES cells in vitro between 4 and 12 days after EB plating. A similar profile was observed in EBs treated with RA during plating (data not shown).

In contrast to the RA-treated groups, cells without RA exposure displayed intensities hardly beyond the 10^3 log and the majority (71-90%) of post-plated EBs were EGFP negative (Fig. 6A, right panel). Additionally, the percentage of EGFP-positive cells increased from 9% (d7+4) to 28% (d7+24) after two to three weeks of plating. This clearly indicated the effect of RA on early onset neural specification and neurogenesis that was otherwise delayed in the untreated ones. It was further corroborated by immunocytochemical findings where the RA-untreated EBs displayed low percentage of MAP2 immunoreactivity after 2-3 weeks of plating (data not shown).

### Functional features of transgenic nestin ES-cell-derived neurons

It is well known that ion channels determine development as well as function of neurons. Therefore, we have focused on the development-dependent expression of ion channels, in particular voltage-activated inward currents. In fact, the live labelling of ES-cell-derived neurons enabled for the first time the investigation of ion channel expression in isolated neurons at very early stages of neurogenesis. Since the differentiation of ES-cell-derived neurons was found to be asynchronous, cells undergoing functional characterization were classified according to the following criteria: (1) time after plating; and (2) morphological characteristics and semi-quantitative assessment of EGFP expression. Isolated neurons from d3-19 after plating were investigated using the classic whole cell patch-clamp technique combined with single cell fluorescence imaging to estimate EGFP intensity. In line with our previous observations, neural progenitors (1804±223, n=25) and unipolar neurons (1585±457, n=25) were characterized by significantly higher EGFP intensities than more differentiated neurons (bipolar neurons, 545±86, n=66; multipolar neurons, 482±77, n=56). Many of the multipolar neurons, probably of a more advanced state of differentiation, were without detectable fluorescence (n=8).

When the expression pattern of ion channels was characterized in the four population of neurons, no voltage-dependent ion currents were detected in neural progenitors/apolar cells during the entire developmental stage (Fig. 7A, n=25). Indeed, at this stage ramp depolarizations as well as single voltage steps (data not shown) did not yield macroscopic currents. EGFP-positive neural progenitors were carefully selected (morphological criteria) in order to avoid contamination by other cell types. The unipolar EGFP-positive neurons (Fig. 7B) expressed voltage-dependent outward rectifying currents (n=7), whereas no voltage-activated inward currents, neither I_Na nor I_Ba, could be detected in the large majority of cells (22 out of 24). The outward rectifying currents recorded in the bipolar as well as multipolar neurons were identified as K+ currents based on their sensitivity towards the blockade by 4-aminopyridine (4-AP). 4-AP (2 mM) inhibited 73.69±8.2% of the aggregate outward current (HP –80 mV, step potential +50 mV) (n=6, data not shown). Interestingly, at the early developmental stage (EDS, d7+3/4), I_Ba but not I_Na (Fig. 7E) was detected in 86% of bipolar neurons (n=7), whereas almost all (80%) of the multipolar neurons (n=5) expressed both I_Na and I_Ba. At subsequent stages (LDS, d7-19) all the bipolar and multipolar neurons functionally expressed both currents. I_Na was further characterized using the Na+ channel selective antagonist TTX which, as expected for neuronal Na+ channels, blocked I_Na completely at a concentration of 0.1 μM (Fig. 7C,D). I_Ba densities in bipolar neurons increased significantly with plating time (Fig. 7F) from 48.1±8.3 to 106.4±10.1 pA/pF (n=60) and in multipolar neurons (data not shown) from 40.4±10.9 to 80.7±7.0 pA/pF (n=50), respectively. I_Ba density in the bipolar neurons was higher than that in the multipolar neurons (Fig. 7G). To further envisage whether there were qualitative and/or quantitative differences in the expression pattern of I_Ba subtypes during development, we investigated both bipolar and multipolar neurons by applying selective antagonists. In four typical experiments of bipolar neurons we found that the L-type Ca2+ channel antagonist Isradipine (3 μM) suppressed 22.7±3.6% of the aggregate I_Ba, the N-type specific Ca2+ channel blocker ω-CgTX (3 μM) suppressed 14.2±3.7% and the P/Q type channel blocker ω-AgaTX (0.1 μM) suppressed 8.5±1.6%, respectively (Fig. 8B,C). The remaining Ca2+, sensitive (50 μM CdCl2) component amounted to 54.6±8.2% of control I_Ba. Similarly, in four typical multipolar neurons the fraction of different Ca2+ channel subtypes amounted to: L-type, 27.3±1.9%; N-type, 9.1±3.2%; P/Q type, 16.2±3.7%; and other-types, 47.4±5.6%, respectively (Fig. 8D).

Since receptor-operated ion channels are known to play an essential role not only in carrying out fast synaptic transmission but also in the mediation of trophic signals, and migration and synaptic arrangement during neuronal development, we tested their functional expression at different time points during neurogenesis. The neural progenitors (n=3) and unipolar neurons (n=4) did not respond to γ-aminobutyric (GABA), glycine, kainate and NMDA. Clear agonist responses to GABA (1 mM) and glycine (1 mM) were detected (Fig. 9A,B) in bipolar (n=8, current density 52.1±22.0 pA/pF) and multipolar neurons (n=8, current density 119.1±51.1 pA/pF). Moreover, as expected, the receptor-operated currents were blocked by the competitive antagonists dibucaine (100 mM, n=8) and strychnine (30 μM, n=8), respectively (HP –80 mV; Fig. 9A,B). To further confirm the ionic nature of the GABA and glycine-evoked currents, voltage ramps in the presence of the agonist were subtracted from control ramps. As can be seen in the inset of Fig. 9A, the subtracted voltage ramps for GABA yielded a reversal potential of –28.3±1.1 mV (n=5), a value close to the calculated Cl- equilibrium potential of –28.3 mV at 35°C. We could also observe kainate responses in bipolar (n=8) as well as multipolar cells (n=7), however current amplitude was low with slow activation kinetics (data not shown).

### Discussion

Nestin as the name spun from neuro-epithelial stem cell has been considered to be an efficient marker for proliferating cells whose expression is downregulated upon differentiation. Hence, examining the nestin intron-II-driven EGFP expression in our ES-cell model helped not only in demarcating the progenitor population and neural subtypes but also in unravelling the quantitative, qualitative and functional neurogenic developmental pattern both prior and subsequent to the onset of neurogenesis.
Fig. 6
Nestin transgenic ES cells and in vitro neurogenesis

1. Nestin transgenic ES cells and in vitro neurogenesis

Nestin expression in transgenic mice was reported to occur as early as stage E7.5 (Zimmerman et al., 1994; Lothian and Lendahl, 1997; Josephson et al., 1998; Lothian et al., 1999; Yaworsky and Kappen, 1999), although it remains unclear whether or not it was investigated at earlier stages. In the transgenic ES-cell model and in murine blastocysts we demonstrated that nestin was expressed before lineage specification. The reported cDNA sequence in the GenBank database from 3.5 dpc murine blastocyst (accession no. C78523) having sequence similarity to mouse, rat, and human nestin genes corroborated our findings on the presence of nestin transcript in vivo. Similar observations on early expression of other tissue-specific cytoskeletal proteins have also been described (Bain et al., 1995). Recently, Streit et al. reported that neural induction in chick embryo occurred before gastrulation, indirectly implicating that nestin, being a neural stem cell marker, is probably evolutionarily conserved and might express prior to gastrulation (Streit et al., 2000). However, the role of nestin, a cytoskeletal protein belonging to the intermediate filament family, prior to the onset of neurulation remains to be determined. It is possible that nestin, being one of the earliest expressing cytoskeletal proteins, is required for laying a strong cytoarchitectural foundation during early embryonic development.

2. Neural ontogeny and nestin expression

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3. Promoter/enhancer targeted neural specification

Previous studies from our group had already demonstrated the efficacy of promoter-mediated targeting in the ES-cell system for a better understanding of cardiomyogenesis (Kolossov et al., 1998). The current investigation allowed us to explore details of in vitro neurogenesis. Previously, Steven Goldman’s group used a promoter-targeted and EGFP-reporter-based transient expression system in primary cultures for neural cell type selection and isolation (Wang et al., 1998; Roy et al., 2000a; Roy et al., 2000b). However, here we took advantage of the versatility of transgenic nestin ES-cell clones combined with stable EGFP expression. The immunocytochemical, electrophysiological and time-lapse observations provided direct proof of the neural lineage confinement of nestin intron-II-driven EGFP expression in
Fig. 8. Functional expression of \(I_{Na}\) during early stages of neural development. (A) The \(I_{Na}\) current-voltage relationship (voltage steps lasting for 150 milliseconds, from –60 to +50 mV in 10 mV increments, HP –80 mV) in a representative bipolar LDS neuronal cell. The threshold of activation was between –60 and –50 mV and peak currents were measured at –10 mV. (B) The fraction of different \(I_{Na}\) subtypes composing the whole cell current was evaluated using selective antagonists. (C,D) The percentage of the different current components did not significantly differ between bipolar- and multipolar neurons.

Neural differentiation and neurogenic quantification

Based on the qualitative microscopic observation, time-lapse monitoring and concurrent quantitative FACS, two distinct temporal nestin induction patterns (lineage- and lineage*), as discerned by EGFP expression, were revealed. The biphasic EGFP expression pattern indeed indicated the decrease in uncommitted ES cells upon differentiation by LIF withdrawal and cell aggregation and concomitant increase in lineage-specified neural progenitors. This indicates that a critical time window exists during this one week regimen for the neural cell fate decision. Hence, selecting these population of nestin-driven, EGFP-expressing ES cells and EB-derived cells through FACS, and conditioning them to a selective lineage such as neurons, astrocytes and oligodendrocytes would further provide us with useful information regarding the guiding cues prior to lineage commitment and specification. Indeed, the time-lapse monitoring of whole EBs (data not shown) as well as of isolated cells unequivocally demonstrated the proliferation, migration and differentiation of EGFP-positive neural progenitors into neurons and glia. Thus, further study in this regard could answer the critical question whether neurons and glia are generated from a common progenitor and the existing crosstalk between these two cell types, as proposed in a number of studies (Tamada et al., 1998; Vernadakis, 1996).

The time course in neurogenic progression revealed that the maximum number of neural progenitors was generated...
Nestin transgenic ES cells and in vitro neurogenesis

Between 4 and 12 days after EB plating. Accordingly, we could subdivide the transgenic EB-derived cells broadly into three groups. The brightest shining, EGFP-positive cells on plated EBs were categorized into group I, which included the subpopulation of mitotically active neural progenitors as revealed by nestin immunostaining and semi-quantitative fluorescence detection in isolated cells. Group II, with medium EGFP, included the population of weak shining bi- and multipolar neurons as well as glia. EGFP-negative cells were categorized under group III, which included the more differentiated neurons and glia along with other non-neural cell types. Hence, the overall heterogeneity in the full length human nestin intron-II-enhancer-driven EGFP expression in ES cells upon differentiation reflects clearly the endogenous nestin expression as reported (Yaworsky and Kappen, 1999) and the asynchrony in neural progenitor generation. Although the significance of this heterogeneity is not well understood, the temporal- and region-dependent differential expression of specific transcription factors leading to neural stem cell heterogeneity might be the causal basis (Yaworsky and Kappen, 1999).

Ion channel expression during neural development

Voltage-dependent ion channels were acclaimed to be cellular fingerprints for the properties and patterns of neuronal cells during differentiation (Takahashi and Okamura, 1998). In earlier studies on the whole EB (Strubing et al., 1995; Arnhold et al., 2000), only cells with distinct neuronal morphology located at the periphery could be characterized. We have taken advantage of single cell isolation and EGFP labelling for the identification and functional characterization; this allowed easy detection of neural progenitors and unipolar neurons that were otherwise almost impossible to discern from non-neural cell types. Based on morphology and EGFP intensity we could distinguish four populations of neuronal cells with different electrophysiological characteristics. The undifferentiated neural progenitors did not display voltage-dependent ion channels, whereas unipolar neurons were found to express voltage-dependent 4-AP sensitive K+ channels. By contrast, differentiated neurons such as bipolar and multipolar neurons expressed voltage-dependent K+ and Ca2+, as well as TTX-sensitive neuronal Na+ channels. Earlier reports (Barish, 1991; Grantyn et al., 1989; Gottmann et al., 1989) in cultured neuronal precursors suggested the expression of low-voltage-activated Ca2+ currents prior to the appearance of voltage-dependent Na+ and Ca2+ currents. However, in line with our findings, Bain et al. reported that, within the first four days of plating, there was a small number of ES-cell-derived neuron-like cells that lacked voltage-activated inward currents, although their differentiation state was not defined (Bain et al., 1995).

The cell-type-specific ion channel expression might serve specific functions during neuronal maturation. It is possible that neurite outgrowth in conjunction with inter-synaptic connections determine the expression of voltage-activated inward currents or vice versa. Neuronal Na+ channels were detected prior to the onset of voltage-dependent Ca2+ channels in differentiating neurons indirectly suggesting the possible existence of two diverse (lagging and leading in terms of ion channel expression), ES-cell-derived bipolar neuronal subtypes. Similar differences in the inception of ICa expression between cultured chick sensory and autonomic neuronal precursors have been reported earlier (Gottmann et al., 1988). Since the Ca2+ entry into neuronal cells through voltage-gated Ca2+ channels influences neuronal excitability as well as synaptic transmission (Augustine et al., 1987; Spitzer et al., 1994), the absence of ICa in unipolar as well as some bipolar neurons at d7+3/4 suggested that these differentiating, relatively young, neurons had not yet established connection with their neighbouring counterparts and hence lacked the functional expression of these ion channels. Pharmacological evaluation of subtypes of voltage-dependent Ca2+ currents in differentiated neurons revealed that these already expressed N- and P/Q-type Ca2+ currents that were expected to be found in neuronal and neuroendocrine cells (Scherubl et al., 1993).
prevalently of the inhibitory type were detected in bipolar and multipolar neurons. Taken together, the expression of functional ion channels in the developing neurons from ES cells in vitro seems to be related not to the stage (day after plating) alone, as proposed earlier (Bain et al., 1995; Strubing et al., 1995), but primarily to the morphology. This demonstrates that ion channel expression parallels closely the cellular functional demands during neurogenesis.

Thus, the use of live reporter EGFP under the regulatory control of the neural-specific enhancer nestin has given us the means to investigate not only its expression, but also quantitative- and functional characteristics of neurogenesis during early stages of development. This system would help to increase the diversity of self-renewing and multipotent neural progenitors by using fluorescence activated cell sorting (FACS) and, consequently, allow their use in experimental transplantation purposes.

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References


