Functional properties of the human ventral mesencephalic neural stem cell line hVM1

Jan Tønnesen [a,1], Emma Gonzalez Seiz [b,1], Milagros Ramos [b], Olle Lindvall [c], Alberto Martinez-Serrano [b], Merab Kokaia [a,*]

[a] Experimental Epilepsy Group, Wallenberg Neuroscience Center, Lund University Hospital, 221 84, Lund, Sweden
[b] Laboratory of Human Neural Stem Cell Biology, Department of Molecular Biology and Center of Molecular Biology Severo Ochoa, U.A.M.-C.S.I.C. Campus Cantoblanco, Madrid, Spain
[c] Laboratory of Neurogenesis and Cell Therapy, Wallenberg Neuroscience Center, Lund University Hospital, 221 84, Lund, Sweden

A B S T R A C T

The human fetal ventral mesencephalon-derived stem cell line, hVM1, yields high number of tyrosine hydroxylase-expressing presumed dopaminergic neurons upon *in vitro* differentiation. Here we report that cells generated from this line differentiate into a neuronal phenotype, express electrophysiological properties of functional neurons and respond to neurotransmitters *in vitro*. However, the electrophysiological properties are immature and the cells require longer maturation time than possible under *in vitro* conditions.

Keywords:
Human neural stem cell
Dopaminergic neuron
In vitro
Electrophysiology
Functional properties
Differentiation
Parkinson's disease

Clinical trials with intrastriatal transplantation of human fetal mesencephalic tissue in patients with Parkinson's disease (PD) have provided proof-of-principle that grafted dopaminergic neurons can reinnervate the denervated striatum, release dopamine, become functionally integrated in host neural circuitries, and in some cases give rise to substantial clinical improvement (Lindvall and Bjorklund, 2004). The quest for a stem cell source has been ongoing ever since in order to provide sufficient quantities of standardized, transplantable dopaminergic neurons to implement clinically competitive cell replacement therapy for PD. Immortalized human neural stem cell lines are suitable model systems for studying genetic and epigenetic influences on stability over passages, and on stem cell proliferation and differentiation *in vitro* and *in vivo*. Their advantage is multipotency within the neural lineage, expandability and stability, making them available over practically indefinite periods of time (Martinez-Serrano et al., 2001; Villa et al., 2002, 2004; Kim, 2007). Several studies have described amelioration of behavioral deficits by grafting such immortalized human stem cells in rodent models of neurological disease (Lee et al., 2007), and have characterized their immunocytochemical and biochemical features. However, few studies have addressed the functional properties of human neural stem cell lines (Jung et al., 1998; Cho et al., 2002; Lotharius et al., 2002; De Filippis et al., 2007; Donato et al., 2007), most of them failing to demonstrate maturation of these cells into neurons that can generate full-blown action potentials (Jung et al., 1998; Cho et al., 2002; De Filippis et al., 2007; Donato et al., 2007).

We recently reported on a newly derived human fetal ventral mesencephalic stem cell line, hVM1, that generates high fractions of dopaminergic cells, even after considerable expansion (>10 passages) (Villa et al., 2009). The hVM1 cell line was subjected to thorough biochemical, genetic, and immunocytochemical characterization to document its usefulness as a model for developing clinically competent cell lines for PD (Villa et al., 2009). Here we have explored the functional properties of hVM1 cells *in vitro*, based on calcium imaging and electrophysiological investigations. Interestingly, using electrophysiological whole-cell patch-clamp measurements, we found neuronal properties in more than half of all recorded cells, including immature action potentials and membrane rectification. We observed generation of mature spontaneous action potentials in one cell. Calcium imaging demonstrated that hVM1 cells, which have differentiated to neurons, are responsive to glutamate, dopamine and GABA, as expected for ventral mesencephalic neurons.

The derivation of the hVM1 cell line was previously described in detail (Villa et al., 2009). Briefly, cells were isolated from the ventral mesencephalic region of a dead, aborted human fetus, aged 10 weeks, at Lund University Hospital, according to guidelines approved by the Lund/Malmö Ethical Committee. Cells were immortalized by infection with a retroviral vector coding for *v-myc* (LTR-vmyc-SV40p-Neo-LTR) (Villa et al., 2000) generating the hVM1 polyclonal cell line (Villa et al., 2009).

* Corresponding author.
E-mail address: merab.kokaia@neurol.lu.se (M. Kokaia).
[1] JT and EGS contributed equally to this study.

Cells were expanded as attached cultures on poly-L-lysine (PLL, 10 μg/ml)-coated dishes in N2 supplemented DMEM/F12 medium in the presence of 20 ng/ml of each of EGF and bFGF (Villa et al., 2009). To induce differentiation of the cells, bFGF and EGF were withdrawn from the medium and human recombinant glial cell line-derived neurotrophic factor (GDNF, 2 ng/ml, Peprotech) and dibutyryl-cAMP (1 μM, Sigma) were added (Lotharius et al., 2002; Riaz et al., 2002). After plating on PLL-coated glass coverslips for differentiation (10^5 cells/cm^2), cells were kept in proliferation medium for 24 h before changing to differentiation medium. All culturing was done at 37 °C and 95% humidity in a low oxygen atmosphere (5% O_2, 5% CO_2). For all experiments, we used hVM1 cell line at passage 11 to 12.

For immunocytochemical analysis, 4% PFA-fixed samples were incubated overnight with primary antibodies against: β-III-tubulin (1:1000, monoclonal, Sigma), Glial Fibrillacy Acidic Protein (GFAP; 1:1000, monoclonal, Sigma) and tyrosine hydroxylase (TH; 1:1000, polyclonal, Chemicon). Cells were then incubated with the secondary antibody for 30 min. Cell nuclei were counterstained with To-Pro (1:500; Invitrogen). Specificities of secondary antibodies were confirmed by parallel immunostainings without the primary antibody.

For calcium imaging experiments, cells were loaded with 10 μM Fura-2 AM (Molecular Probes) for 30 min at 37 °C in HBSS medium added 5.55 mM glucose and 0.02% Pluronic F-127 (Molecular Probes), and washed for 20 min in HBSS medium. Next, coverslips were mounted in a perfusion chamber on the microscope stage at 37 °C as described in Ruiz et al. (1998). Single cell analysis of the changes in cytosolic calcium concentration ($[Ca^{2+}]_i$) was expressed as the ratio of Fura-2 fluorescence intensity at 340 and 380 nm excitation. Different neurotransmitters and potassium were added as a bolus at the indicated times and at the following concentrations (arrows in Figs. 1D, G, L): 100 μM dopamine (Sigma); 100 μM γ-aminobutyric acid (GABA, Tocris Cookson); 100 μM glutamate (Sigma). The concentrations of drugs and potassium are within the range previously reported to elicit cytosolic calcium release in calcium imaging experiments (Jung et al., 1998; Malmersjo et al., 2009). Cells were washed with Mg-free HBSS medium and supplemented with 30 μM of glycine just prior to the addition of glutamate. Two selective receptor antagonists were used: 5-methyl-10,11-dihydro-5-dibenzocyclohepten-5,10-imine maleate (MK-801; 50 μM; Tocris Cookson) for blocking glutamate receptors (NMDA subtype) and picrotoxin (PTX; 100 μM; Tocris Cookson) for blocking $GABA_A$ receptors. For KCl-induced depolarization of cells, the recording chamber was perfused with an isosmotic HBSS solution containing 60 mM KCl (Supplementary figure 1).

Functional properties of differentiated stem cells were evaluated by the whole-cell patch-clamp technique as described previously (Parish, Castelo-Branco et al. 2008). In short, coverslips with differentiated cells were transferred to a recording chamber continuously perfused at a rate of 3 ml/min, at room temperature, with carbogenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) containing: NaCl 119 mM; KCl 2.5 mM; $MgSO_4$ 1.3 mM; $CaCl_2$ 2.5 mM; $NaHCO_3$ 26.2 mM; NaH_2PO_4 1 mM; glucose 11 mM (300 mOsm, pH 7.4). Patch-clamp recording pipette tip resistances were 3.5–5 MΩ when filled with solution containing K-gluconate 122.5 mM; KCl 17.5 mM; NaCl 8 mM; KOH-HEPES 10 mM; KOH-EGTA 0.2 mM; MgATP 2 mM; and Na_3GTP 0.3 mM (295 mOsm, pH 7.2). Resting membrane potential was measured in current clamp mode at 0 pA. Input resistance was assessed from 5 mV square voltage pulses at RMP. For I/V curves, we measured the membrane potential, while injecting 500 ms positive or negative currents stepwise (Figs. 1M, N). Whole-cell currents and voltages were amplified by a patch-clamp amplifier (EPC10; HEKA Elektronik).

All data are presented as mean ± standard error of the mean (SEM). Comparisons of parameters within the cell lines were done by Student's *t*-test, and analysis for temporal changes within the cell lines by linear regression. Level of significance was set at $p<0.05$.

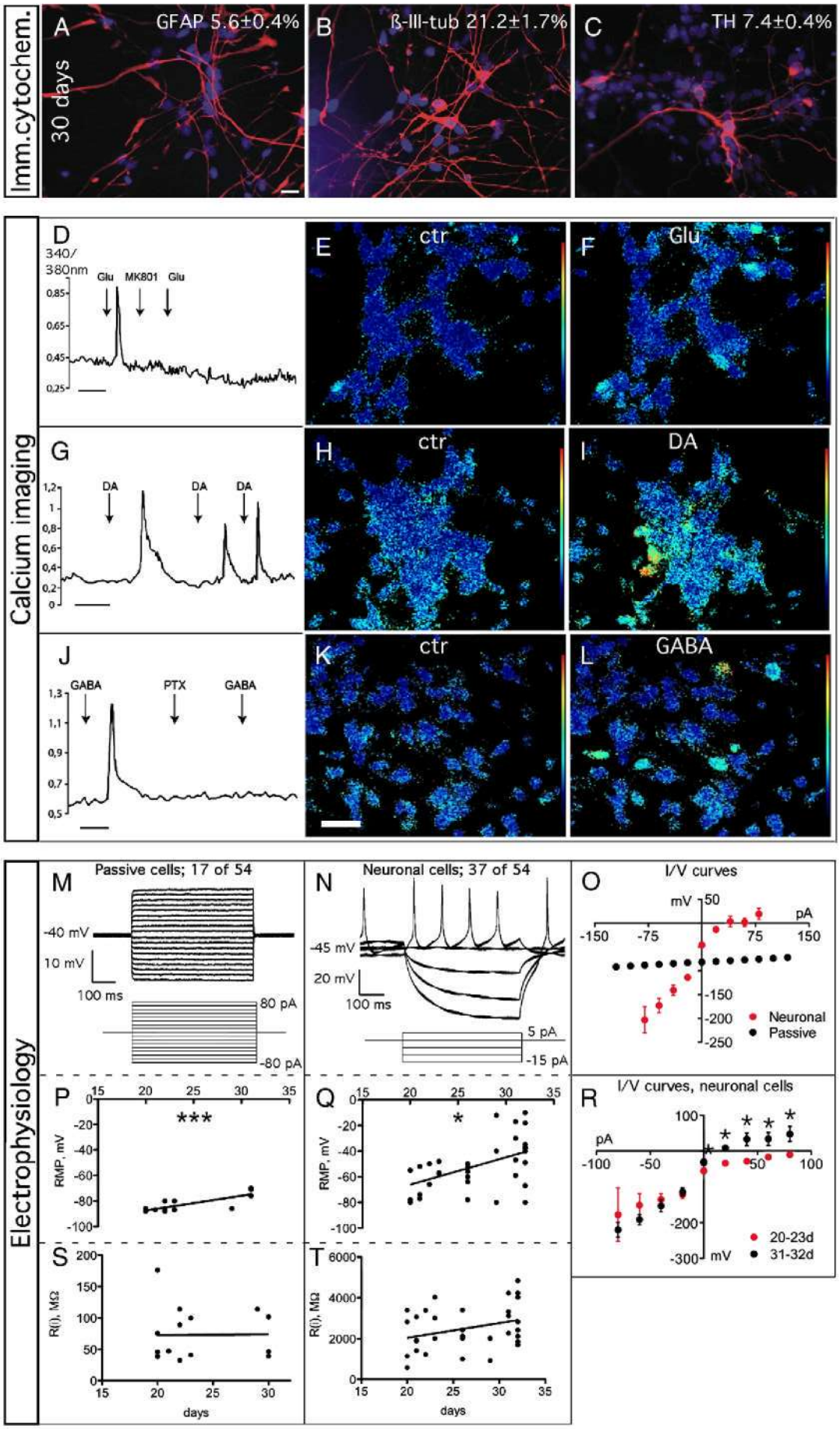
Expression of the neuronal marker β-III-tubulin and the dopaminergic marker TH reached maximum after 12 days under differentiation conditions, and was observed in 19.7 ± 1.6% and 12.1 ± 1.0% of the total number of cells, respectively. The GFAP+ glial cell population at this stage of differentiation constituted 2.9 ± 0.3% of all cells (Supplementary figure 1A-C). After 30 days of differentiation, the percentage of TH-expressing cells decreased compared to 12 days to 7.4 ± 0.4%, while the percentage of GFAP+ cells increased to 5.6 ± 0.4%. The fraction of β-III-tubulin expressing cells remained stable at 30 days (21.2 ± 1.7% of the total population) and did not differ from that at 12 days (Figs. 1A–C).

After 12 days of differentiation, application of 60 mM potassium increased intracellular calcium in 9.5 ± 2.4 % of cells (Supplementary figure 1D-F). Application of 100 μM dopamine elicited a calcium increase in 8.8 ± 1.9% of cells, while 9.6 ± 2.7% and 7.3 ± 1.7% of cells responded to 100 μM glutamate and 100 μM GABA, respectively (Figs. 1D–L), already after 7 days of differentiation. Glutamate responses were blocked by the NMDA receptor antagonist MK-801, and GABA responses by the $GABA_A$ receptor antagonist PTX (Figs. 1D, J), confirming receptor specificity.

Using whole-cell patch-clamp recordings at later stages of differentiation (between 20 and 32 days), we found that 37 out of 54 recorded cells (68%) displayed active membrane properties suggestive of an immature neuronal phenotype (Fig. 1N). Sixteen cells (43%) fired immature action potentials upon current injections. Over the differentiation period, there was a significant decrease in resting membrane potential (RMP) assessed as linear regression and with *t*-test, from −65.4 ± 4.5 mV at days 20–21 (n = 8) to −41.6 ± 7.3 mV at day 32 ($n=9$; Figs. 1Q, R). This was not accompanied by significant changes in input resistance (R_i) (assessed by linear regression and *t*-test), the average value at days 20–21 being 2029 ± 353 ($n=8$) and at day 32 being 3003 ± 425 ($n=8$; Fig. 1T). We further compared the current–voltage (*I–V*) relationship of the recorded cells at days 20–23 with those at days 31–32. There was clearly less inward rectification during application of hyper- and depolarizing pulses to the membrane in the cells at days 31–32 of differentiation, as compared to days 20–22 (Fig. 1R). Among 16 cells exhibiting passive membrane properties (Fig. 1M), we observed a significant decrease in RMP, from −86.4 ± 0.76 mV at days 20–22 ($n=10$) to −75.8 ± 3.7 mV at days 29–32 ($n=4$; Fig. 1P). The R_i was unaltered in these cells over time, being 69.6 ± 14.6 MΩ at days 20–22 ($n=10$) and 75.4 ± 19.0 MΩ at days 29–32 ($n=4$, Fig. 1S). The nominally high resting membrane potential, the lack of rectification and the low R_i indicate that these cells have acquired glial-like properties.

The hVM1 cell line is able to survive in differentiation medium for more than 30 days, and from days 20–32 of differentiation more than half of the cells expressed neuronal electrophysiological properties, including membrane rectification and immature action potential firing. In one cell, we observed spontaneous action potentials that increased in frequency upon current injection-induced depolarization. These findings provide evidence that expanded immortalized human fetal neural stem cells sustain their molecular identity to acquire neuronal properties, and that differentiation can be achieved *in vitro*. It should

Fig. 1. Immunocytochemical analysis after 12 days of differentiation revealed 5.6 ± 0.4% GFAP positive cells (A), 21.2 ± 1.7% β-III-tubulin expressing cells (B) and 7.4 ± 0.4 % TH positive cells (C). Calcium imaging confirmed that the hVM1 cell could respond to bolus application of Glutamate (D–F), dopamine (DA) (G–I) and GABA (J–L), and that the response to glutamate could be blocked by the NMDA receptor antagonist MK-801 (D), while the GABA response was blocked by the $GABA_A$ antagonist PTX (J). Electrophysiological patch-clamp recordings confirmed neuronal and glial subpopulations of cells, that could be discriminated based on passive membrane properties in the glial fraction (M, O) and active properties, including rectification and action potential firing, in the neuronal fraction (N, O). Among both neuronal and glial cells there was a decrease in the resting membrane potential over the recording period, covering days 19–31 of differentiation (P, Q). The input resistance of the cells did not change over this period (S, T). Loss of membrane rectification over time (R), together with decrease in membrane potential, in the neural population, indicates compromised cell membrane. Scale bar in D, G, J: 2 min; in A and K: 20 μm. *$p<0.05$.


Imm.cytochem.
A
GFAP 5.6±0.4%
30 days
B
ß-III-tub 21.2±1.7%
C
TH 7.4±0.4%
Calcium imaging
D
340/
380nm
Glu
MK801
Glu
E
ctr
F
Glu
G
DA
DA
DA
H
ctr
I
DA
J
GABA
PTX
GABA
K
ctr
L
GABA
Electrophysiology
M
Passive cells; 17 of 54
-40 mV
10 mV
100 ms
80 pA
-80 pA
N
Neuronal cells; 37 of 54
-45 mV
20 mV
100 ms
5 pA
-15 pA
O
I/V curves
mV
pA
Neuronal
Passive
P
RMP, mV
***
Q
RMP, mV
*
R
I/V curves, neuronal cells
pA
mV
20-23d
31-32d
S
R(i), MΩ
days
T
R(i), MΩ
days

be noted, though, that we did not observe post-synaptic currents in any of the recorded cells under the present conditions, indicating that no functional synaptic connections between cells had been established.

Our finding that maximum expression of β-III-tubulin occurs after 12 days and is unchanged after 30 days of differentiation corroborates the original report describing the immunocytochemical properties of the hVM1 cell line (Villa et al., 2009). In this previous report we additionally confirmed expression of an array of midbrain dopaminergic markers, including GIRK2, Calretinin, DAT, VMAT2, ADH2, Lmx1a, Lmx1b, Pitx3, Nurr-1, En1, Mash1 and Ngn2, pointing to a substantia nigral profile of these cells (Villa et al., 2009).

Over the reported time period, we observed a relative increase in GFAP-expressing glial cells and decrease in the fraction of TH-expressing cells. These data indicate a loss of TH-expressing catecholaminergic neurons and possibly also other mature neurons, concomitant with the increase in the glial population. The decline in percentage of neurons was also suggested by the electrophysiological data, showing a reduction of RMP among both neuronal and passive cells over time, accompanied by a decrease in inward rectification in the neuronal population, indicating a compromised cell membrane. The gradual loss of membrane integrity likely reflects shortcomings in the *in vitro* culturing conditions.

More than half of the recorded cells expressed immature neuronal properties. This neuronal fraction is substantially higher than that estimated based on the presented immunocytochemical data (20% of cells express β-III-tubulin), and from calcium imaging data (7–10% of cells respond to glutamate, dopamine or GABA). This discrepancy could be due to bias in visually choosing cells with more neuronal appearance for patch-clamp recordings. However, it could also reflect higher sensitivity of the electrophysiological approach, compared to immunocytochemistry, as a method for evaluating neuronal differentiation.

We found that GABA application, similar to glutamate, induced calcium inflow into the cells. Normally, GABA hyperpolarizes neurons, driving the membrane potential away from what would activate voltage-dependent calcium channels (Rabow et al., 1995). However, in immature neurons, GABA acts as depolarizing transmitter due to higher chloride concentration inside the cells as compared to outside (Cherubini et al., 1991; Rivera et al., 1999). This was apparently the case in our experiments with calcium imaging.

Dopamine can depolarize or hyperpolarize neurons through D1 and D2 metabotrobic receptors, respectively. The D2 receptors are expressed on nigral dopaminergic neurons mediating autoinhibition (Lacey et al., 1989). Recent experiments have demonstrated D2 autoreceptor activation induces intracellular calcium release in human embryonic stem cell-derived dopaminergic neurons at all stages of maturation (Malmersjo et al., 2009). Therefore, it is likely that the observed increase in intra-cellular calcium of hVM1 cells in response to dopamine application is a D2-mediated response in dopaminergic neurons. Alternatively, it can be a D1 receptor mediated response in non-dopaminergic neurons, expressing D1 receptors. Further studies are needed to elucidate the expression and function of dopamine receptor subtypes in hVM1 cells.

The hVM1 cell line may become useful for generating dopaminergic, and possibly other types of neurons, for experimental cell therapy approaches. It should be pointed out, though, that *v-myc* immortalization of these cells is compromising with respect to their potential clinical applications (Nilsson and Cleveland, 2003). Our data provide evidence that the multipotent hVM1 human ventral mesencephalic stem cell line is capable of differentiating into functional neurons, although under the present *in vitro* conditions with relatively immature properties. It was not possible to retrospectively identify recorded cells as dopaminergic under the current conditions. However, our previous immunocytochemical data show that 47% of β-III-tubulin expressing cells were TH-positive, i.e., presumably dopaminergic (Villa et al., 2009). Therefore, it is reasonable to suggest that a considerable fraction of the recorded cells with immature neuronal properties were dopaminergic. Future *in vivo* studies, enabling longer survival times, and the development of human dopaminergic reporter constructs, e.g. human-TH-GFP, will shed light on the question whether these cells can differentiate to fully mature and functional ventral mesencephalic dopaminergic neurons.

Acknowledgments

This publication was supported by the European Community's Seventh Framework Programme grants Excell (Nr. 214706) and NeuroStemcell (Nr. 22943). Work at AMS group was supported by grants from Spanish Ministry of Science and Innovation (BIO2007-66807 and PLE2009-0101) and Carlos III Institute of Health (RETICS TerCel RD06/0010/0009). The Institutional grant from Foundation Ramón Areces to the Center of Molecular Biology Severo Ochoa (CBMSO) is also gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.expneurol.2010.01.013.

References

Cherubini, E., Gaiarsa, J.L., et al., 1991. GABA: an excitatory transmitter in early postnatal life. Trends Neurosci. 14 (12), 515–519.
Cho, T., Bae, J.H., et al., 2002. Human neural stem cells: electrophysiological properties of voltage-gated ion channels. Neuroreport 13 (11), 1447–1452.
De Filippis, L., Lamorte, G., et al., 2007. A novel, immortal, and multipotent human neural stem cell line generating functional neurons and oligodendrocytes. Stem Cells 25 (9), 2312–2321.
Donato, R., Miljan, E.A., et al., 2007. Differential development of neuronal physiological responsiveness in two human neural stem cell lines. BMC Neurosci. 8, 36.
Jung, M., Kramer, E.M., et al., 1998. Novel pluripotential neural progenitor lines exhibiting rapid controlled differentiation to neurotransmitter receptor-expressing neurons and glia. Eur. J. Neurosci. 10 (10), 3246–3256.
Kim, S.U., 2007. Genetically engineered human neural stem cells for brain repair in neurological diseases. Brain Dev. 29 (4), 193–201.
Lacey, M.G., Mercuri, N.B., et al., 1989. Two cell types in rat substantia nigra zona compacta distinguished by membrane properties and the actions of dopamine and opioids. J. Neurosci. 9 (4), 1233–1241.
Lee, H.J., Kim, K.S., et al., 2007. Brain transplantation of immortalized human neural stem cells promotes functional recovery in mouse intracerebral hemorrhage stroke model. Stem Cells 25 (5), 1204–1212.
Lindvall, O., Bjorklund, A., 2004. Cell therapy in Parkinson's disease. NeuroRx 1 (4), 382–393.
Lotharius, J., Barg, S., et al., 2002. Effect of mutant alpha-synuclein on dopamine homeostasis in a new human mesencephalic cell line. J. Biol. Chem. 277 (41), 38884–38894.
Malmersjö, S., Liste, I., Dyachok, O., Tengholm, A., Arenas, E., Uhlen, P., 2009. Ca2+ and cAMP signaling in human embryonic stem cell-derived dopamine neurons. Stem Cells and Development (Electronic publication ahead of print). doi:10.1089/scd.2009.0436.
Martinez-Serrano, A., Rubio, F.J., et al., 2001. Human neural stem and progenitor cells: in vitro and in vivo properties, and potential for gene therapy and cell replacement in the CNS. Curr. Gene Ther. 1 (3), 279–299.
Nilsson, J.A., Cleveland, J.L., 2003. Myc pathways provoking cell suicide and cancer. Oncogene 22 (56), 9007–9021.
Parish, C.L., Castelo-Branco, G., et al., 2008. Wnt5a-treated midbrain neural stem cells improve dopamine cell replacement therapy in parkinsonian mice. J. Clin. Invest. 118 (1), 149–160.
Rabow, L.E., Russek, S.J., et al., 1995. From ion currents to genomic analysis: recent advances in GABAA receptor research. Synapse 21 (3), 189–274.
Riaz, S.S., Jauniaux, E., et al., 2002. The controlled conversion of human neural progenitor cells derived from foetal ventral mesencephalon into dopaminergic neurons in vitro. Brain Res. Dev. Brain Res. 136 (1), 27–34.
Rivera, C., Voipio, J., et al., 1999. The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature 397 (6716), 251–255.
Ruiz, F., Alvarez, G., et al., 1998. Protection by pyruvate and malate against glutamate-mediated neurotoxicity. Neuroreport 9 (7), 1277–1282.
Villa, A., Liste, I., et al., 2009. Generation and properties of a new human ventral mesencephalic neural stem cell line. Exp. Cell Res. 315 (11), 1860–1874.
Villa, A., Navarro, B., et al., 2002. Genetic perpetuation of in vitro expanded human neural stem cells: cellular properties and therapeutic potential. Brain Res. Bull. 57 (6), 789–794.
Villa, A., Navarro-Galve, B., et al., 2004. Long-term molecular and cellular stability of human neural stem cell lines. Exp. Cell. Res. 294 (2), 559–570.
Villa, A., Snyder, E.Y., et al., 2000. Establishment and properties of a growth factor-dependent, perpetual neural stem cell line from the human CNS. Exp. Neurol. 161 (1), 67–84.