Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 309 (2003) 91–95
BBRC
www.elsevier.com/locate/ybbrc

Early neuronal progenitor cell line expressing solely non-catalytic isoform of TrkC☆

Janusz Matuszyk, Ewa Ziolo, Dagmara Plawiak, and Leon Strzadala*

Laboratory of Cellular Immunology, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw 53-114, Poland

Received 23 July 2003

Abstract

TrkC is a receptor for neurotrophin-3 that regulates development of neuronal precursors. Transduction of signals into receptor-dependent signaling pathways is mainly due to the activation of the intrinsic tyrosine kinase of the TrkC receptor. Alternative splicing of the trkC transcripts generates catalytic and non-catalytic isoforms. The non-catalytic isoform, denoted as TrkC-NC2, contains unique sequence, instead of deleted entire kinase domain. Here, we report that neural cell line MB-G, derived from brain of embryos of transgenic tsA58-SV40 mice, contains mRNA encoding TrkC-NC2 without concomitant expression of mRNA for catalytic TrkC molecule.

Keywords: Tyrosine kinase; Truncated receptor; Neuron-restricted precursor; Transgenic mice

The neurotrophins regulate proliferation, differentiation, and apoptosis of neuronal precursors during embryonic development [1]. The neurotrophins act through Trk receptors that bind specific neurotrophic factor: TrkA binds nerve growth factor (NGF), TrkB binds brain-derived neurotrophic factor (BDNF) and NT-4/5, and TrkC binds neurotrophin-3 (NT-3). Signaling pathways regulated by TrkC are necessary for survival of a subset of neurons during development of the peripheral nervous system, especially those of sensory neurons involved in proprioception [2]. TrkC-dependent signaling pathways also play an important role in the development of some cells outside the nervous system. It has been shown that mice with targeted deletion of TrkC exhibit severe cardiac defects, suggesting that TrkC-dependent signaling pathways also regulate development of the mammalian heart [3]. Finally, expression of TrkC receptors was found in promyelocytes, myelocytes, megacaryocytes, and other cells in human normal bone marrow, suggesting that TrkC-mediated signaling could play a role in differentiation of specific hematopoietic progenitors [4].

The trkC gene encodes a number of differentially spliced isoforms with distinct capabilities for signal transduction. Demonstration of diverse functional properties of TrkC isoforms suggests that alternative splicing can regulate the activation of signaling pathways leading to survival and differentiation of neural cells [5–9]. Alternative splicing of the trkC transcripts can generate non-catalytic, truncated TrkC isoforms, lacking the cytoplasmic tyrosine kinase domain. At least five cDNAs encoding different non-catalytic TrkC isoforms have been described in mammals and chicken [6–8,10]. The most abundant non-catalytic isoform, denoted as TrkC-NC2 in mouse [10] and TrkC(ic158) in rat [7], contains unique sequence, highly conserved among species, instead of deleted entire kinase domain. In the adult brain mRNAs encoding TrkC-NC2 were widely expressed in different structures together with mRNAs for tyrosine kinase-containing receptor (TrkC-K) [10]. The physiological role of TrkC-NC2 remains to be uncovered. It was suggested [10] that truncated TrkC proteins could inhibit kinase-dependent signaling

☆ *Abbreviations:* GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; NF-L, neurofilament light chain; NT-3, neurotrophin-3; SV40-Tag, simian virus 40 large T antigen; TrkC-K, catalytic tyrosine kinase-containing isoforms of TrkC; TrkC-NC2, non-catalytic isoform 2 of TrkC.

* Corresponding author. Fax: +48-71-337-1382.
E-mail address: strzadal@iitd.pan.wroc.pl (L. Strzadala).

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-291X(03)01501-8

pathways by forming heterodimer with TrkC-K. On the other hand, it was shown by Hapner et al. [11] that ectopically expressed truncated TrkC receptor together with a p75 neurotrophin receptor promoted neuronal differentiation of avian neural crest cells following stimulation with NT-3. This observation suggests that truncated isoform of TrkC might activate kinase-independent signaling pathways in response to NT-3.

Establishment of immortalized neural cell lines [12–19] has made the opportunity to study the process of neural differentiation at a molecular level. Here, we report that mouse embryo brain-derived neural cell line may contain mRNA for TrkC-NC2 without concomitant expression of mRNA for TrkC-K.

Materials and methods

Animals. Transgenic mice tsA58-SV40 (Immortomouse) harboring transgene encoding temperature-sensitive mutant tsA58 of SV40 large T antigen (SV40-Tag) under the control of the γ-interferon-inducible H-2Kb promoter [20] were gifted from Dr. H. von Boehmer, Institut Necker, Paris, France. Adult animals were anesthetized with Forane and killed by cervical dislocation according to NIH guidelines. Embryos were removed and decapitated.

Cell culture. MB-G cell line was derived from mouse brains of three embryos, at embryonic day 15–16, of tsA58-SV40 transgenic mice. Briefly, cells were isolated from brains with the use of syringe needle and mechanically dissociated. Cells were cultured at 33 °C (permissive temperature for activity of SV40-Tag) in flasks (Corning Costar, Corning, NY, USA) coated with poly-L-lysine, in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS, Boehringer–Mannheim, Germany), 4.5 g/L glucose, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% supernatant containing γ-interferon. During passages by mild trypsinization, cells having long processes were eliminated from the cell culture. Further cells were grown in the medium supplemented with 10% FCS, 10% supernatant containing recombinant γ-interferon, and 10% supernatant obtained from concanavalin A-stimulated mouse splenocytes.

Rat hippocampal progenitor cell line H19-7 (gift from Dr. E. Eves, Ben May Institute for Cancer Research, The University of Chicago, USA) was generated from embryonic day 17 hippocampal cells of Holtzman rat embryos [15]. They were conditionally immortalized by stable transfection with temperature-sensitive (U19tsa mutation) SV40-Tag. They were grown at 33 °C in flasks coated with 15 μg/ml of poly-L-lysine (Sigma) in DMEM supplemented with 10% FCS, 4.5 g/L glucose, 50 U/ml penicillin, 50 μg/ml streptomycin, and 200 μg/ml G418.

Immunocytochemistry. Cells were fixed in Chamber Slide-8 well glass slide (Nunc-LabTek) with 4% formalin in phosphate-buffered saline (PBS) for 30 min at 37 °C, washed with PBS, and incubated for 1 h at room temperature with permeabilization buffer containing 0.15% Triton X-100 in PBS with 10% of horse serum and subsequently washed with PBS. Next, cells were incubated for 18 h with monoclonal anti-βIII tubulin antibody (Promega, Madison, WI, USA) diluted at 1:200 in permeabilization buffer. RNA was removed from preparations by incubation for 30 min with 1 mg/ml of RNase. After washing with PBS, cells were incubated for 1 h with 2 μM YO-PRO-1 iodide (Molecular Probes) and Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) diluted at 1:1000 in permeabilization buffer. Following labeling, preparations were mounted using the ProLong Antifade Kit (Molecular Probes). Fluorescence imaging was performed using two-color confocal scanning laser microscopy (Bio-Rad MRC 1024) and the laser power was attenuated to 3% of maximal output power. Cell nuclei stained with YO-PRO-1 appeared green while tubulin βIII antigen stained with Cy3 was red under the 488 nm excitation.

Reverse transcriptase-polymerase chain reaction. Total RNA was isolated using Trizol reagent (Invitrogen) from cultures of cells and from mouse brain. Genomic DNA was removed from preparations of RNA with the use of DNase I. Aliquots 10 μg of the total RNA samples were used to synthesize first-strand cDNA using 200 U of SuperScript II (Invitrogen) reverse transcriptase (RT) and 1 μg of oligo(dT)12–18 primers in a 20 μl final reaction volume for 75 min at 41 °C. The polymerase chain reaction (PCR) were performed on

Table 1
Primers used for the RT-PCR

Primer	Sequence	Product (bp)
HPRT	F 5′-CCTGCTGGATTACATTAAAGCACTG-3′ R 5′-CCTGAAGTACTCATTATAGTCAAGG-3′	370
Tubulin βIII	F 5′-CCTTCGATTCCCTGGTCAGCTC-3′ R 5′-TGGGCTTCCGATTCCTCGTC-3′	624
MAP2	F 5′-AGAACCAATTCGCAGAGCAGGA-3′ R 5′-GGCCTGTGACGGATGTTCTTTAGA-3′	408
α-Internexin	F 5′-GAGCCAGCCGAGAGGAGATC-3′ R 5′-ACTGGGATTGGGCAGTGGAT-3′	356
NF-L	F 5′-TCTTCGGCCGTTCTGCTTAC-3′ R 5′-CTTCCTCCTCACCCTCACCA-3′	300
GFAP	F 5′-GCTGGAGGCAGAGAACAACCTG-3′ R 5′-TGGCGGCGATAGTCGTTAGC-3′	378
TrkC-K (1)	F-5′ GCCTCCCAGCACTTTGTGCA-3′ R 5′-CCAGCACCCCAGCATGACAT-3′	369
TrkC-K (2)	F-5′ CCACTTCCTGAAGGAGCCCT-3′ R 5′-CACTCAGCCAGGAAGACCTT-3′	492
TrkC-K (3)	F 5′-CCACTTCCTGAAGGAGCCCT-3′ R 5′-TGGGCCCTGAGGAACTTGTT-3′	717
TrkC-NC2	F 5′-CCACTTCCTGAAGGAGCCCT-3′ R 5′-CCCACTCTGGACCTCAGGTT-3′	517

Primers for HPRT were drawn from [25]; reverse primer for GFAP was drawn from [26].

amount of cDNA corresponding to 1/25, 1/250, and 1/2500 parts of serially diluted volume after reaction with reverse transcriptase. PCR was performed in a total volume of 20 μl containing cDNA, 0.4 μM each of 5′ and 3′ primers, and 0.5 U *Taq* polymerase (Sigma). The sequences of oligonucleotides used as primers are shown in Table 1. PCRs with HPRT primers were performed to test the amount and quality of cDNAs. Samples were subjected to 35 cycles (for tested genes) or 30 cycles (for HPRT) of PCR according to the following scheme: 94 °C for 45 s, 60 °C (for tested genes) or 56 °C (for HPRT) for 45 s, and 72 °C for 1 min. The absence of contaminating genomic DNA in the RT-PCR analysis was demonstrated with parallel RT reactions from the same RNA in which the reverse transcriptase was omitted. Total brain RNA was used in the RT-PCR as a positive control. The amplified products were resolved on 2% agarose gels and visualized by ethidium bromide staining under UV illumination.

Results

MB-G cells express neuron-specific tubulin βIII protein

In a preliminary experiments, using primers complementary to the sequence encoding extracellular domain of TrkC, RT-PCRs were performed with cDNAs of cells isolated from brains of 15- to 16-day-old embryos of transgenic tsA58-SV40 mice and maintained in different culture conditions. For more detailed study we have selected mouse brain-derived neural cells, denoted as MB-G, which expressed mRNA encoding TrkC receptor. The cells appeared flat, polygonal or elongated in shape, with short or long processes (Fig. 1). Next, MB-G cells and H19-7 cells were stained immunocytochemically for tubulin βIII, a neuron-specific marker. Immortalized rat hippocampal H19-7 cells were used as reference cell line expressing markers indicative of commitment to neuronal lineage. Using confocal microscopy, the expression of tubulin βIII protein was found in both MB-G and H19-7 cells, whereas C6 glioma cells that served as negative control remained unstained with anti-tubulin βIII antibody (Fig. 2 shows result for MB-G).

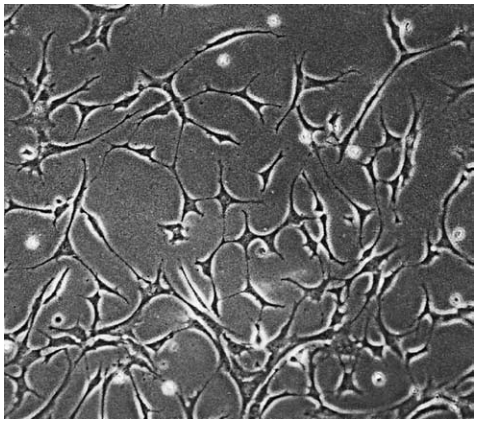

Fig. 1. Phase-contrast photomicrograph of MB-G cells cultured at 33 °C.

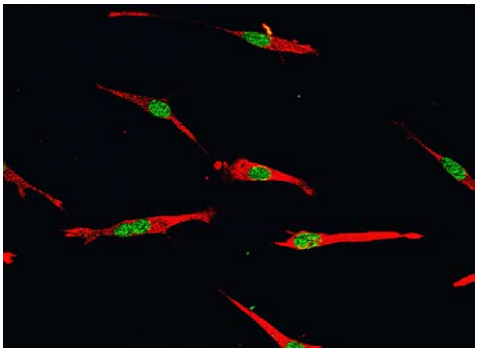

Fig. 2. Expression of neuron-specific tubulin βIII protein in MB-G cells. Cells were immunostained for tubulin βIII (red) with monoclonal anti-βIII tubulin antibody plus Cy3-conjugated donkey anti-mouse IgG, stained for nuclei (green) with YO-PRO-1 iodide, and analyzed by confocal microscopy. Specificity of the protein expression was confirmed by lack of staining of C6 glioma cells with anti-βIII tubulin antibody (not shown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

MB-G and H19-7 cells express mRNA encoding neuron-specific cytoskeletal proteins

RT-PCR assays were performed to determine expression of genes encoding neuron-specific cytoskeletal proteins. Using specific oligonucleotides complementary to the analyzed sequences yielded the expected size of amplified fragments when PCRs were carried on total RNA of mouse brain (positive control). We first confirmed the presence of mRNA encoding tubulin βIII in MB-G and H19-7 cells (Fig. 3A). Results indicate that both MB-G and H19-7 cells express the mRNA and the protein of tubulin βIII, well-known neuron-specific antigenic marker expressed in the neuronal precursors and their progeny. Next, we examined expression of genes encoding microtubule-associated protein 2 (MAP2), α-internexin, and neurofilament light chain (NF-L), proteins appearing later than tubulin βIII during the development of neurons [21]. Expression of mRNA for MAP2 was found both in MB-G and H19-7 cells (Fig. 3A). PCRs with primers complementary to the mRNAs encoding α-internexin and NF-L yielded faint product of amplification of cDNA fragment for α-internexin but not for NF-L in both MB-G and H19-7 cells (Fig. 3A). On the other hand, it was found that mRNA encoding the glial fibrillary acidic protein (GFAP, marker of astrocytes) was not detected by RT-PCR using total RNA from different cultures of MB-G cells (Fig. 3B).

Taken together, MB-G as well as H19-7 cells have mRNAs encoding tubulin βIII and MAP2, neuron-specific markers appearing in neuronal precursors termed neuron-restricted precursors [22]. Note that the mRNA level for MAP2 was repeatedly lower in MB-G cells than in H19-7 cells. Transcript for NF-L, late appearing marker of neurons, was absent in MB-G and H19-7 cells.

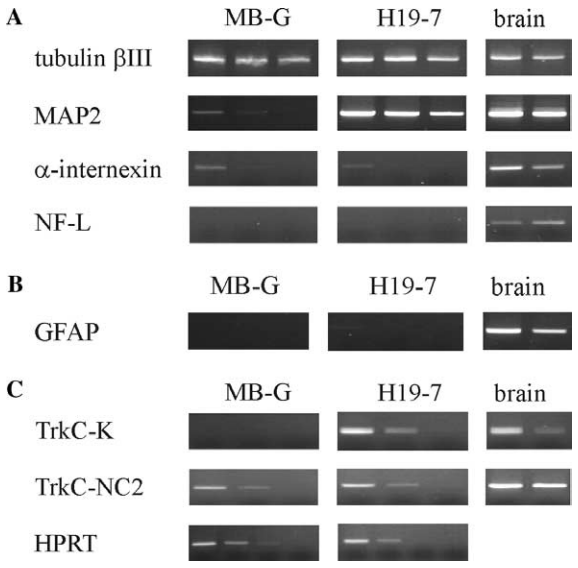

Fig. 3. RT-PCR assays for mRNAs encoding cytoskeletal proteins and isoforms of TrkC in MB-G cells and H19-7 cells. The PCRs were performed on cDNAs serially diluted (1/25, 1/250, and 1/2500). Brain cDNA was used as positive control of primers. HPRT primers were used to test the amount and quality of cDNAs. Results are representative of several experiments using cells from three different refrozen samples. (A) Neuron-specific markers: tubulin βIII, MAP2, α-internexin, and NF-L. (B) Glial-specific marker GFAP. (C) mRNAs for catalytic (TrkC-K) and non-catalytic (TrkC-NC2) isoforms of TrkC.

Differential expression of transcripts encoding catalytic and truncated isoforms of TrkC in MB-G and H19-7 cells

Finally, the MB-G and H19-7 cells were tested by RT-PCR with the use of four pairs of primers (see Table 1) for the expression of catalytic (TrkC-K) and non-catalytic (TrkC-NC2) isoforms. H19-7 cells were found to express mRNAs encoding both the TrkC-K and TrkC-NC2 isoforms (Fig. 3C). In contrast, MB-G cells were shown to express mRNA for TrkC-NC2 without concomitant expression of transcripts encoding TrkC-K (Fig. 3C).

Discussion

Here we have compared expression of transcripts encoding catalytic and non-catalytic isoforms of TrkC receptor in two cell lines, MB-G and H19-7, derived from developing brain and immortalized with SV40-Tag. Both MB-G and H19-7 cells, when cultured at 33 °C (temperature permissive for the activity of SV40-Tag), express early (tubulin βIII, MAP2) but not late (NF-L) appearing markers of neurons. It was shown here that H19-7 cells express transcript for non-catalytic isoform (TrkC-NC2) together with transcript for catalytic isoform (TrkC-K) of TrkC receptor. On the other hand, MB-G cells were found to express mRNA on clearly detectable level only for TrkC-NC2. Transcript for TrkCK was absent in MBG cells or it was barely detected in some RT-PCRs.

TrkC-K was detected in undifferentiated HiB5 multipotent neural precursor cell line [23]. Moreover, results of Menn et al. [24] have indicated that whilst TrkC-K isoforms were expressed both in mitotic and postmitotic cells, TrkC-NC2 was present only in differentiating neural stem cell progeny. As major issue we have presented here results showing that cells with phenotype of the neuron-restricted precursors may express mRNA encoding TrkC-NC2 without accompanying expression of mRNA for TrkC-K.

Acknowledgments

We thank Dr. E. Eves, Ben May Institute for Cancer Research, The University of Chicago, USA for her gift of H19-7 cells, Ms. B. Gidzinska, Ms. J. Kozlak and Dr. D. Dus for analyses using confocal microscopy. This work was supported by the Grant No. 4P05A06819 from the Polish State Committee for Scientific Research and partially by the Grant No. 3/2001 from the Polish Academy of Sciences.

References

[1] D.R. Kaplan, F.D. Miller, Neurotrophin signal transduction in the nervous system, Curr. Opin. Neurobiol. 10 (2000) 381–391.
[2] R. Klein, I. Silos-Santiago, R.J. Smeyne, S.A. Lira, R. Brambilla, S. Bryant, L. Zhang, W.D. Snider, M. Barbacid, Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements, Nature 368 (1994) 249–251.
[3] L. Tessarollo, P. Tsoulfas, M.J. Donovan, M.E. Palko, J. Blair-Flynn, B.L. Hempstead, L.F. Parada, Targeted deletion of all isoforms of the trkC gene suggests the use of alternate receptors by its ligand neurotrophin-3 in neuronal development and implicates trkC in normal cardiogenesis, Proc. Natl. Acad. Sci. USA 94 (1997) 14776–14781.
[4] E. Labouyrie, P. Dubus, A. Groppi, F.X. Mahon, J. Ferrer, M. Parrens, J. Reiffers, A. de Mascarel, J.P. Merlio, Expression of neurotrophins and their receptors in human bone marrow, Am. J. Pathol. 154 (1999) 405–415.
[5] F. Lamballe, P. Tapley, M. Barbacid, trkC encodes multiple neurotrophin-3 receptors with distinct biological properties and substrate specificities, EMBO J. 12 (1993) 3083–3094.
[6] P. Tsoulfas, D. Soppet, E. Escandon, L. Tessarollo, J.L. Mendoza-Ramirez, A. Rosenthal, K. Nikolics, L.F. Parada, The rat trkC locus encodes multiple neurogenic receptors that exhibit differential response to neurotrophin-3 in PC12 cells, Neuron 10 (1993) 975–990.
[7] D.M. Valenzuela, P.C. Maisonpierre, D.J. Glass, E. Rojas, L. Nunez, Y. Kong, D.R. Gies, T.N. Stitt, N.Y. Ip, G.D. Yancopoulos, Alternative forms of rat TrkC with different functional capabilities, Neuron 10 (1993) 963–974.
[8] A.S. Garner, T.H. Large, Isoforms of the avian TrkC receptor: a novel kinase insertion dissociates transformation and process outgrowth from survival, Neuron 13 (1994) 457–472.
[9] P. Tsoulfas, R.M. Stephens, D.R. Kaplan, L.F. Parada, TrkC isoforms with inserts in the kinase domain show impaired signaling responses, J. Biol. Chem. 271 (1996) 5691–5697.
[10] B. Menn, S. Timsit, G. Calothy, F. Lamballe, Differential expression of TrkC catalytic and noncatalytic isoforms suggests that they act independently or in association, J. Comp. Neurol. 401 (1998) 47–64.

[11] S.J. Hapner, K.L. Boeshore, T.H. Large, F. Lefcort, Neural differentiation promoted by truncated trkC receptors in collaboration with p75(NTR), Dev. Biol. 201 (1998) 90–100.
[12] P.F. Bartlett, H.H. Reid, K.A. Bailey, O. Bernard, Immortalization of mouse neural precursor cells by the c-myc oncogene, Proc. Natl. Acad. Sci. USA 85 (1988) 3255–3259.
[13] E.F. Ryder, E.Y. Snyder, C.L. Cepko, Establishment and characterization of multipotent neural cell lines using retrovirus vector-mediated oncogene transfer, J. Neurobiol. 21 (1990) 356–375.
[14] P.J. Renfranz, M.G. Cunningham, R.D. McKay, Region-specific differentiation of the hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain, Cell 66 (1991) 713–729.
[15] E.M. Eves, M.S. Tucker, J.D. Roback, M. Downen, M.R. Rosner, B.H. Wainer, Immortal rat hippocampal cell lines exhibit neuronal and glial lineages and neurotrophin gene expression, Proc. Natl. Acad. Sci. USA 89 (1992) 4373–4377.
[16] M.F. Mehler, R. Rozental, M. Dougherty, D.C. Spray, J.A. Kessler, Cytokine regulation of neuronal differentiation of hippocampal progenitor cells, Nature 362 (1993) 62–65.
[17] S.R. Whittemore, L.A. White, Target regulation of neuronal differentiation in a temperature-sensitive cell line derived from medullary raphe, Brain Res. 615 (1993) 27–40.
[18] M. Hoshimaru, J. Ray, D.W. Sah, F.H. Gage, Differentiation of the immortalized adult neuronal progenitor cell line HC2S2 into neurons by regulatable suppression of the v-myc oncogene, Proc. Natl. Acad. Sci. USA 93 (1996) 1518–1523.
[19] Y. Qi, J.K. Wang, M. McMillian, D.M. Chikaraishi, Characterization of a CNS cell line, CAD, in which morphological differentiation is initiated by serum deprivation, J. Neurosci. 17 (1997) 1217–1225.
[20] P.S. Jat, M.D. Noble, P. Ataliotis, Y. Tanaka, N. Yannoutsos, L. Larsen, D. Kioussis, Direct derivation of conditionally immortal cell lines from an H-2Kb-tsA58 transgenic mouse, Proc. Natl. Acad. Sci. USA 88 (1991) 5096–5100.
[21] M.P. Kaplan, S.S. Chin, K.H. Fliegner, R.K. Liem, M.P. Kaplan, S.S. Chin, K.H. Fliegner, R.K. Liem, Alpha-internexin, a novel neuronal intermediate filament protein, precedes the low molecular weight neurofilament protein (NF-L) in the developing rat brain, J. Neurosci. 10 (1990) 2735–2748.
[22] M.S. Rao, Multipotent and restricted precursors in the central nervous system, Anat. Rec. 257 (1999) 137–148.
[23] V.L. Sheen, M.W. Arnold, Y. Wang, J.D. Macklis, Neural precursor differentiation following transplantation into neocortex is dependent on intrinsic developmental state and receptor competence, Exp. Neurol. 158 (1999) 47–62.
[24] B. Menn, S. Timsit, A. Represa, S. Mateos, G. Calothy, F. Lamballe, Spatiotemporal expression of noncatalytic TrkC NC2 isoform during early and late CNS neurogenesis: a comparative study with TrkC catalytic and p75NTR receptors, Eur. J. Neurosci. 12 (2000) 3211–3223.
[25] R. Pernas-Alonso, F. Morelli, U. di Porzio, C. Perrone-Capano, Multiplex semi-quantitative reverse transcriptase-polymerase chain reaction of low abundance neuronal mRNAs, Brain Res. Brain Res. Protoc. 4 (1999) 395–406.
[26] G.L. Colucci-D'Amato, A. Tino, R. Pernas-Alonso, J.M. ffrench-Mullen, U. di Porzio, Neuronal and glial properties coexist in a novel mouse CNS immortalized cell line, Exp. Cell. Res. 252 (1999) 383–391.