

High neuronal/astroglial differentiation plasticity of adult rat hippocampal neural stem/progenitor cells in response to the effects of embryonic and adult cerebrospinal fluids

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Summary

Hippocampal neural stem/progenitor cells (hipp-NS/PCs) of the adult mammalian brain are important sources of neuronal and glial cell production. In this study, the main goal is to investigate the plasticity of these cells in neuronal/astroglial differentiations. To this end, the differentiation of the hipp-NS/PCs isolated from 3-month-old Wistar rats was investigated in response to the embryonic cerebrospinal fluid (E-CSF) including E13.5, E17-CSF and the adult cerebrospinal fluid (A-CSF), all extracted from rats. CSF samples were selected based on their effects on cell behavioral parameters. Primary cell culture was performed in the presence of either normal or high levels of KCL in a culture medium. High levels of KCL cause cell depolarization, and thus the activation of quiescent NSCs. Results from immunocytochemistry (ICC) and semi-quantitative RT-PCR (sRT-PCR) techniques showed that in E-CSF-treated groups, neuronal differentiation increased (E17>E13.5). In contrast, A-CSF decreased and increased neuronal and astroglial differentiations, respectively. Cell survivability and/or proliferation (S/P), evaluated by an MTT assay, increased by E13.5 CSF, but decreased by both E17 CSF and A-CSF. Based on the results, it is finally concluded that adult rat hippocampal proliferative cells are not restricted progenitors but rather show high plasticity in neuronal/astroglial differentiation according to the effects of CSF samples. In addition, using high concentrations of KCL in the primary cell culture led to an increase in the number of NSCs, which in turn resulted in the increase in neuronal or astroglial differentiations after CSF treatment.

Key words: Hippocampal neural stem/progenitor cells, Embryonic CSF, Adult CSF, Neuronal/astroglial differentiation

Introduction

There are two primary neurogenic regions of the adult mammalian brain: the subventricular zone (SVZ) of lateral ventricles (Lois and AlvarezBuylla, 1993) and the subgranular layer (SGL) of the hippocampus (Altman and Das, 1965).

Proliferative cells in the hippocampus include neural stem and progenitor cells (NS/PCs) which are the cause of neuron turnover of the hippocampus throughout life as it is in the subventricular zone (SVZ), (Altman and Das, 1965; Reynolds and Weiss, 1992; Palmer *et al.*, 1995, 1997; Garcia *et al.*, 2004; Schmetsdorf *et al.*, 2005).

Proliferative cells from the adult rat hippocampus have been demonstrated to be multipotent and responsive to fibroblast growth factor 2 (FGF-2) (Palmer *et al.*, 1995). However, others have reported that the adult rodent hippocampal dentate gyrus contains only restricted progenitors (Seaberg and van der Kooy, 2002). Bull and Bartlet (2005) demonstrated that the characteristics of bona fide neural stem cells including large sphere diameter, long-term self-renewal and neuronal differentiation were not clearly observed in those of the hippocampus. Using depolarizing levels of extracellular KCL *in vitro*, Walker *et al.* (2008)

suggested that the rodent hippocampus possesses quiescent NSCs which can be activated by depolarizing, resulting in the production of large and long-term self-renewal neurospheres.

Many *in vivo* studies have shown that NS/PCs can be modulated by different factors, particularly those coming from other parts of the brain contained in the CSF (Miyan *et al.*, 2003). Several researches have suggested important roles for CSFs in neurodevelopmental events through affecting NS/PCs during both embryonic period and adulthood in vertebrates (Miyan, 2003; Gato *et al.*, 2005; Buddensiek *et al.*, 2009; Martin *et al.*, 2009; Buddensiek *et al.*, 2010). *In vitro* studies have shown that E-CSF and A-CSF both have strong effects on cell proliferation and differentiation in an age-dependent manner, and the effects depend on the age of the animal from which the CSF is extracted (Buddensiek *et al.*, 2009, 2010; Martin *et al.*, 2009; Nabiumi *et al.*, 2012; Carnicero *et al.*, 2013).

The authors of the present work hypothesize that assessing E-CSF or A-CSF effects on proliferative cells could help understand whether rodent hippocampal NS/PCs cells are highly plastic in differentiation. Thus, we noted the effects of E13.5, E17 and A-CSF on NS/PCs in accordance with the experiments mentioned

above. As proteomic studies have demonstrated great similarity in the composition and biological functions of proteins present in the E-CSF of humans and rats (Zappaterra *et al.*, 2007), we expect the same similarity for A-CSF. We also used depolarizing levels of KCL to activate quiescent NSCs (Walker *et al.*, 2008) aiming at comparing them with normal cultures. What is reported in this study is how E-CSF including E13.5, E17, and A-CSF affect behavioral parameters of hipp-NS/PCs, and whether these cells have high differentiation plasticity when differentiated into neuronal and astroglial cells as bona fide NSCs.

Materials and Methods

Obtaining embryonic and adult cerebrospinal fluid

Wistar rats were mated overnight and sperm-positive vaginal smears were detected the following morning. This day was considered as embryonic day 0 (E0). E13.5 and E17 embryos were explanted and dissected from extra-embryonic membranes. E-CSF from the cisterna magna cavity was aspirated as previously described by Miyani *et al.* (2006). Adult 6-month-old Wistar rats were anesthetized and the A-CSF from the cisterna magna cavity was aspirated as previously described by Liu and Duff, (2008). CSF samples were aliquoted, frozen in liquid nitrogen and stored at -80°C (Burgos *et al.*, 2013).

Isolation of neural stem/progenitor cells from hippocampal dentate gyrus

An NSC isolation protocol was used as described by Walker *et al.* (2007) and Hagihara *et al.* (2009). Adult 3-month-old rats were sacrificed and their brains were dissected from the skull. The dentate gyrus was then separated from hippocampal formations and chopped into small fragments. For enzymatically digestion, the tissue fragments were incubated in Trypsin-EDTA 0.1% for 7 min, when enzyme activity was neutralized. The digested fragments were centrifuged at 100 *rcf* for 7 min. The pellet was resuspended in 1 ml of expansion medium and filtered through a 40 µm cell sieve (Falcon, BD Biosciences, Bedford, MA). The expansion medium comprised of DMEM/F12 (2:1) containing 100 U/ml penicillin/streptomycin, 2 µg/ml Heparin, growth factors (GFs) EGF and FGF-2, both 20 ng/ml (all from Sigma, St. Louis, MO). To activate quiescent NSCs additional KCL was added to the expansion medium to reach a final concentration of 15 mM (Walker *et al.*, 2008). Primary hipp-NS/PCs were cultured in 25-cm² flasks containing 5 ml of expansion medium containing either normal or 15 mM KCL. The cells were incubated for 10 d in humidified 5% CO₂ at 37°C to permit neurosphere formation after which neurospheres were passaged and cells from both groups were separately expanded in the expansion medium with a normal KCL concentration. We used the cells after three passages but not more to avoid contamination of primary cells and loosing proliferative cell population. The latter can be observed progressively following more than 3 passages (Bull and

Bartlett, 2005).

Proliferation conditions

After the third passage, we cultured cells from either normal or high concentrations of KCL-treated primary cell (n/h-KPC) cultures in the expansion medium or DMEM/F12 without GFs but containing CSF (15% or 20%) for 24 h after which the cell S/P was quantitatively determined by the MTT assay as described later in this article.

Differentiation conditions

After the third passage, we plated hipp-NS/PCs from n/h-KPC cultures on poly-L-lysine (PLL) coated 24-well plates (Nunc, America) in DMEM/F12 without GFs but containing either 7% fetal calf serum (FCS) as control (Gato *et al.*, 2005) or CSF (20%). Cells were allowed to differentiate for 7 days.

MTT assay

The MTT assay previously described by Nabiuni *et al.* (2012) was used for the hipp-NS/PCs from n/h-KPC cultures expanded in an expansion medium or DMEM/F12 without GFs but containing CSF (15% or 20%). All experiments were carried out in 96-well culture plates. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetra-zoliumbromide) is a yellow tetrazolium dye which is reduced by Reductase enzymes in living cells from a pale yellow color to dark blue formazan crystals. Cells were incubated with MTT (5 mg/mL in PBS) for 3 h at 37°C. To make Formazan crystals soluble, DMSO (dimethylsulfoxide) was added to each well. The absorbance of the formazan was then determined at a wavelength of 570 nm using a plate reader (ELx800, Vienna/Austria).

Immunostaining

Immunocytochemistry was carried out as previously described (Buddensiek *et al.*, 2010). To sum up, cell cultures were fixed in 4% paraformaldehyde. Cells were stained for neuronal and astroglial markers (MAP2&GFAP) using an FITC-conjugated secondary antibody. Cell nuclei were stained with 4,6-diamidino-2-phenyl indole (DAPI) for counterstaining. Photomicrographs were captured using a fluorescent microscope (Olympus BX51, US).

Gene expression analysis by sRT-PCR

Total RNA extraction was performed using an RNx-Plus solution (RN7713C, Sinagene, Tehran, Iran). To perform semi-quantitative RT-PCR (sRT-PCR), RNA samples were quantitated, and an equal quantity of each sample (50 ng) was used to be reversely transcribed (RT) into cDNA using a cDNA Synthesis Kit containing a Random primer (Fermentase, Vilnius, Lithuania). Then using a PCR master mix (Sinagene, Tehran, Iran) and genes-specific primers (Table 1), the PCR reaction was carried out using a thermal cycler (Azco Biotech, Inc., CA, USA) with an initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 30 s, annealing (at

Table 1: Gene-specific primers designed and used in sRT-PCR

Gene (protein)	Accession No.	Product length (bp)	Annealing T (°C)	Sequences (forward, reverse)
Nestin	NM_012987	214	62	CCACTGGGATTTCCAGGAGC CTGGAGACCTCAGGGACTCC
Sox2	NM_001109181	417	60	TTACCTCTTCCTCCCACTC CCTCCAGATCTCTCATAAAAAGT
Ki67	NM_139186.2	500	60	AGGATCTGACGCAAATACAACGG GGAAGGACCAGTGAAGGAAAACATAT
β3-tubulin	AF459021.1	414	60	CCCGGGTTAAAGTCCTTCAGT CCAACCTAAACTGATCCCCAGG
Gfap	NM_017009.2	443	60	CGGGATGGCGAGGTCATTAAG TGTCTTGCTCCAGCAGCCTA

temperatures given in Table 1) for 40 s and extension at 72°C for 1 min in 35 cycles. This was followed by a final extension step at 72°C for 10 min. These parameters were in accordance with PCR standard protocols.

Acquisition of gel images and quantitative analysis

RNA samples and PCR products were subjected to electrophoresis in 2% agarose gel which was then stained in 0.5 µg/ml ethidium bromide. Images of the stained agarose gel were acquired via a gel documentation system (G. Box, England). Quantification of the bands was performed by Labimage software (V2.6, Kapelan, Germany).

Cell count and statistical analysis

To quantify the percentage of cells expressing a given marker, the number of positive cells of 5 representative areas per experiment was determined relative to the total number of DAPI-labeled nuclei. A total number of 500 to 1,000 cells were counted per marker. The mean values of 3 experiments for each condition are given together with standard deviations (SDs). Statistical comparisons were made by running an ANOVA and a t-test. All values are expressed as means ± standard errors of the mean (SEM). $P < 0.05$ was considered as statistically significant.

Results

Cell morphology

Cell morphology investigations showed that in stemness and proliferative states, the hipp-NS/PCs formed neurospheres floating in the culture medium and not attaching to the culture dishes (Fig. 1A). After differentiation, the cells were attached to the bottom of the culture dishes coated by PLL and showed interactions among them (Fig. 1B).

Expression of proliferative and NS/PC markers

The sRT-PCR analysis showed that markers *ki67* (proliferative), *nestin* and *sox2* (stemness) were highly expressed in hipp-NS/PCs, depending on KCL concentration. We also observed much lower levels of β3-tubulin and *Gfap* expressions as neuronal and astroglial markers respectively. However, we did not consider this low level of expression as prominent

contamination or impurification of the cell population (Figs. 2A, B).

The effects of E-CSF and A-CSF on the cell S/P of Hipp-NS/PCs

Results from the MTT assay showed that the cell S/P of hipp-NS/PCs decreased and increased in response to E17 CSF, A-CSF, and E13.5 CSF, respectively. We also found that the effect of CSF on the cell S/P depends on the amount of CSF in the culture medium (Fig. 3).

The elevated expression of MAP2 and GFAP in Hipp-NS/PCs from high concentrations of KCL-treated primary cell cultures

After the differentiation process, hipp-NS/PCs from both N&H-KPC cultures showed that 14.44% ± 5.35% and 17.82% ± 2.12% of the cells were GFAP⁺ astrocytes, respectively. The expression level of MAP2 was higher than that of GFAP; 21.56% ± 3.92% and 36.21% ± 2.2%, respectively. These non-CSF-treated groups were considered as control groups (Figs. 4A-C).

Induced neuronal differentiation by E13.5-CSF and E17-CSF without any strong effect on astroglialogenesis

Our results showed that hipp-NS/PCs were highly neurogenic in response to E13.5 and E17-CSF. Normal-KCL-E13.5-CSF-and-E17-CSF-treated groups showed that 40.96% ± 5.35% and 71.93% ± 5.08% of the cells were MAP2⁺ neurons. However, 23.05% ± 1.1% and 9.74% ± 3.11% were GFAP⁺ found to be astrocytes, respectively. High KCL-E13.5-CSF-treated-and-E17-CSF-treated groups showed higher levels of expression in the MAP2 marker (58.02% ± 3.1% and 82.12% ± 5.1%, respectively). The expression of the GFAP marker increased in high KCL-E13.5-CSF-treated groups (34.21% ± 2.1%) but decreased in E17-CSF-treated groups (7.11% ± 2.2%) (Figs. 4A, B).

Gliogenesis promotion and neurogenesis inhibition by A-CSF in hipp-NS/PCs

We allowed the hipp-NS/PCs to differentiate in the presence of A-CSF as described in the previous section. The results showed significant increases and decreases in GFAP and MAP2 expressions, respectively. Hipp-NS/PCs from N&H-KPC cultures treated with A-CSF showed that 78.23% ± 2.13% and 99.12% ± 3.1%

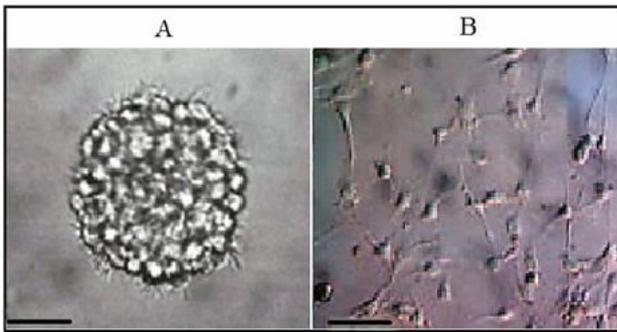


Fig. 1: Cell morphology. A) Growing neurospheres after second passage. B) Differentiation of NS/PCs after 7 days (scale bars = 50 μm)

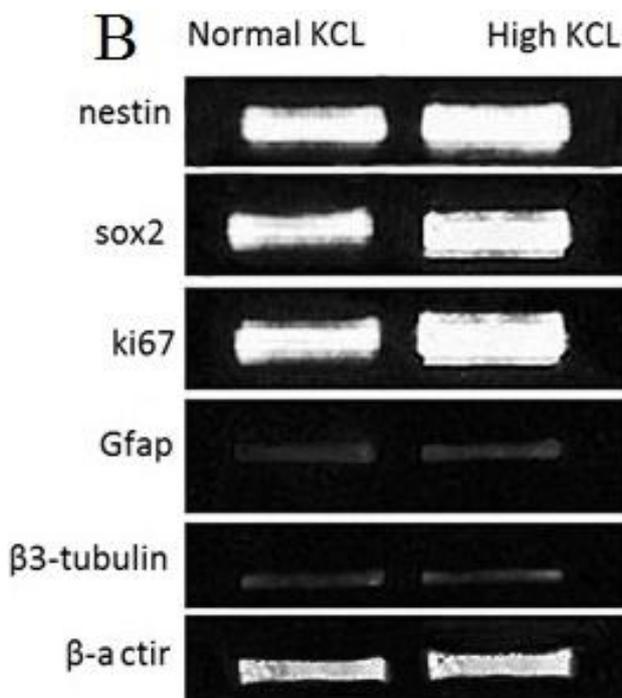
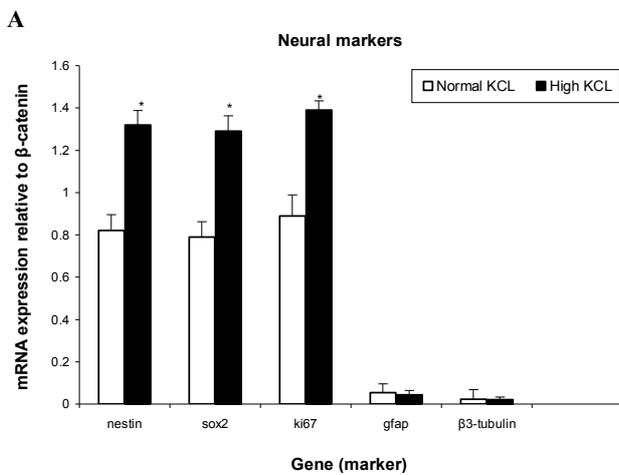


Fig. 2: sRT-PCR analysis of gene expression in NS/PCs from either normal or high KCL-treated cultures before differentiation process. A) Stemness and proliferative markers (Nestin, Sox2, and Ki67, respectively). All were compared with the expression of housekeeping gene β-actin. Values represent means ± SEMs. B) Gel electrophoresis of PCR products of neural markers using 2% agarose gel in a TBE buffer

of the cells were GFAP⁺. In contrast, 14.76% ± 5.1% and 11.32% ± 5.1% of the cells were MAP2⁺ (Figs. 4A, B).

Gene expression affected by CSF during differentiation

Before doing sRT-PCR, we showed all RNA samples

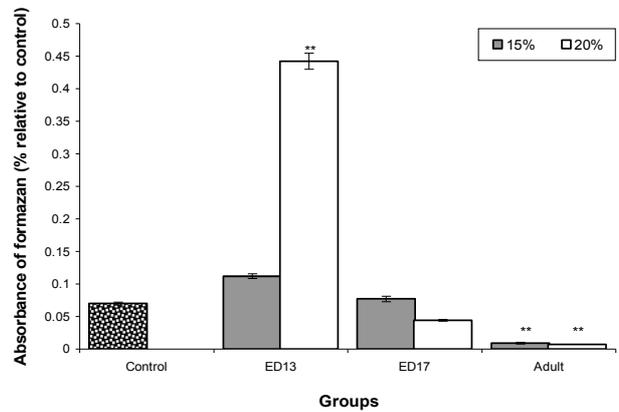
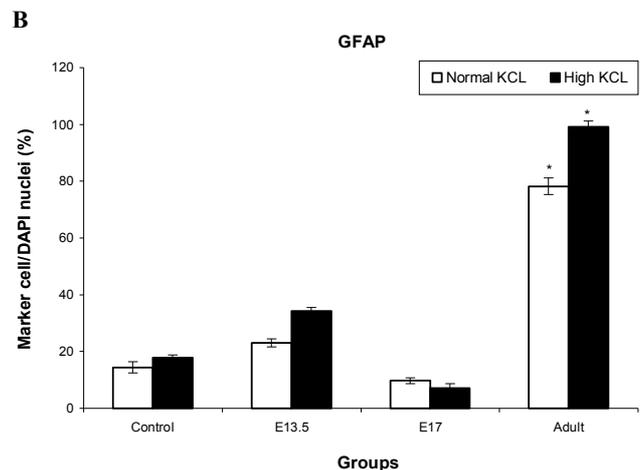
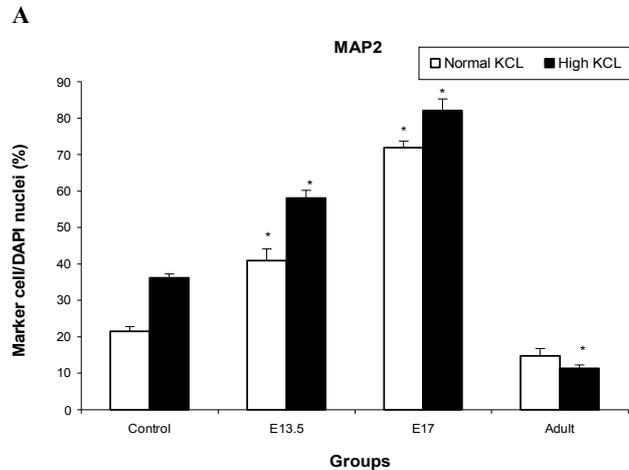


Fig. 3: Survival and/or proliferation of NS/PCs after culture in expansion medium or DMEM/F12 (without GFs) containing E-CSF (E13.5&E17) or A-CSF (both 15% and 20%) for 24 h. Reduction of MTT was measured colourimetrically by the absorbance of formazan products. All results were compared with the control. Values represent means ± SEMs from at least three independent experiments. * P<0.05, and ** P<0.01



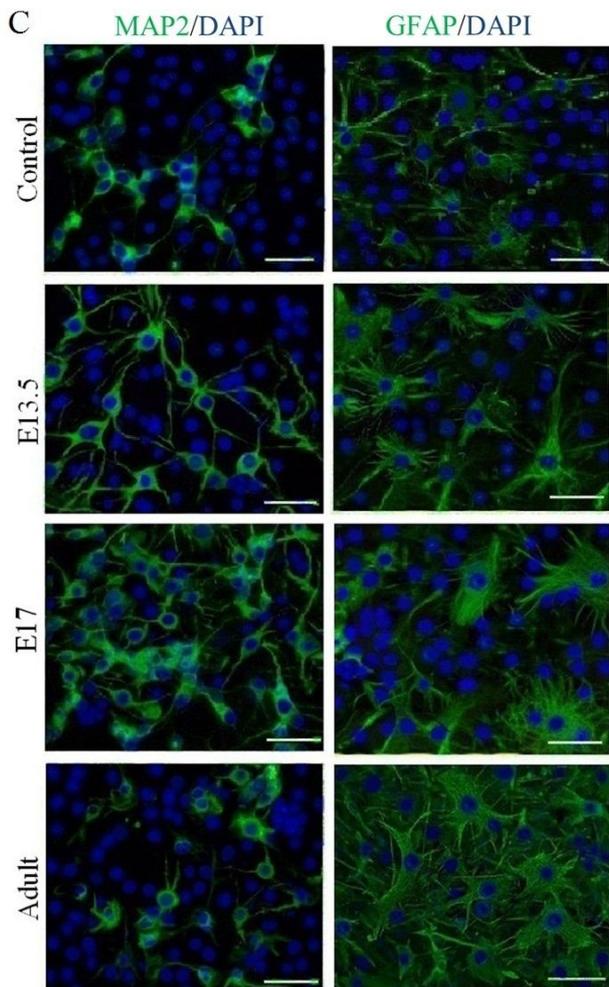


Fig. 4: Immunocytochemical analysis of gene expression. A, B) Quantitative data of MAP2 and GFAP expression obtained by staining NS/PCs from either normal or high KCL-treated cultures differentiated for 7 days in DMEM/F12 without GFs but containing either 7% FCS (control) or 20% CSF including E-CSF (E13.5, E17) and A-CSF. Results are mean values \pm SEMs from at least three independent experiments (* $P < 0.05$). C) Representative microphotographs showing differentiated NS/PCs expressed MAP2 and GFAP with the counterstaining of nuclei by DAPI (scale bars = 10 μ m)

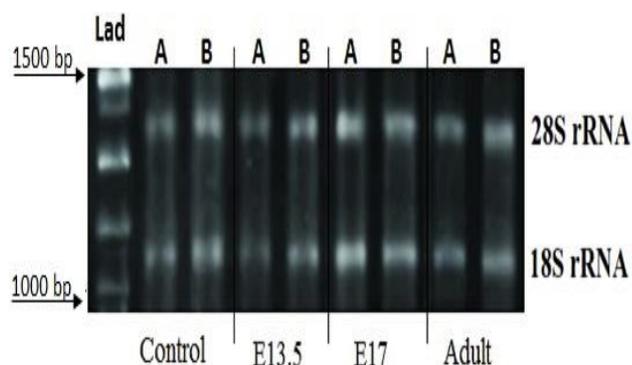
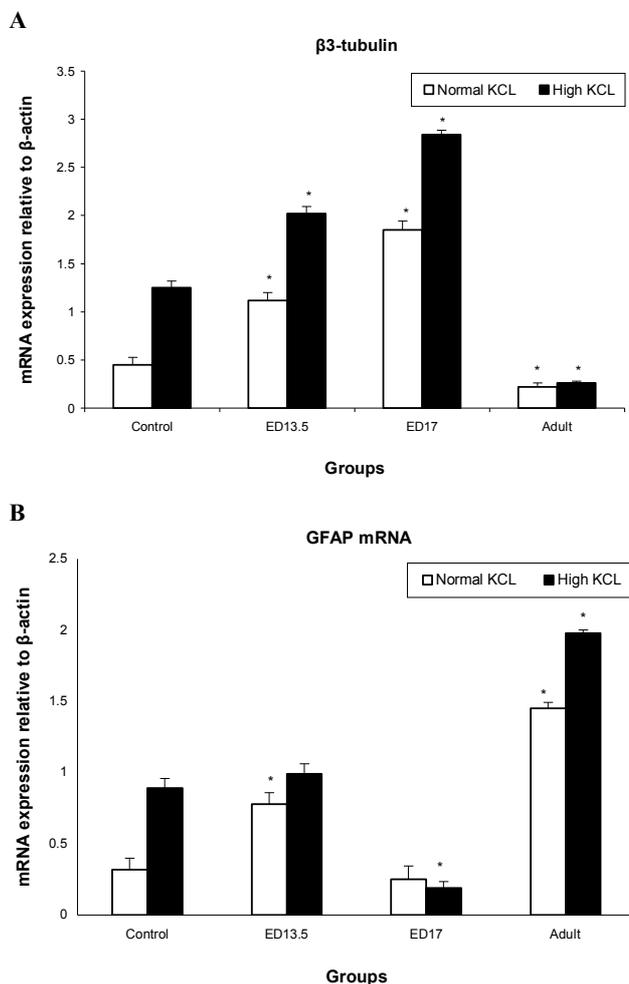


Fig. 5: RNA electrophoresis from each treatment group of the differentiation test using 2% agarose gel in a TBE buffer. 18 and 28 rRNA bands were clearly detected. A) Normal KCL, and B) High KCL. Lad: Ladder

were perfect (Fig. 5). Results from the gene expression analysis by sRT-PCR showed high gene expression levels of the markers *Nestin*, *Sox2* and *Ki67* before differentiation when the markers *Gfap* and β 3-tubuline were expressed in a very low level (Figs. 2A, B). Seven days after starting the differentiation process, the expression of *Nestin* and *Ki67* dropped (data not shown). In contrast, the expression of *Gfap* (in A-CSF-treated group) and β 3-tubulin (in E13.5-and-E17-treated groups) significantly increased (Figs. 6A, B). The electrophoresis images have been showed in Figs. 6C-F.

Discussion

The purpose of the present work was to find out whether rodent hipp-NS/PCs are highly plastic in neuronal/astroglial differentiation by testing the effects of E-CSF and A-CSF on these cells. Results from ICC and sRT-PCR methods showed that these cells highly differentiate into neuronal and astroglial cells in response to E13.5/E17-CSF and A-CSF, respectively. It has been reported that rat E13.5-CSF induces cell proliferation and neuronal differentiation in neuroepithelium isolated from rat embryos (Martin *et al.*, 2009). Miyan (2006) and Nabiuni (2012) have independently showed that E17-CSF strongly causes neuronal differentiation but decreases cell proliferation in embryonic neural stem



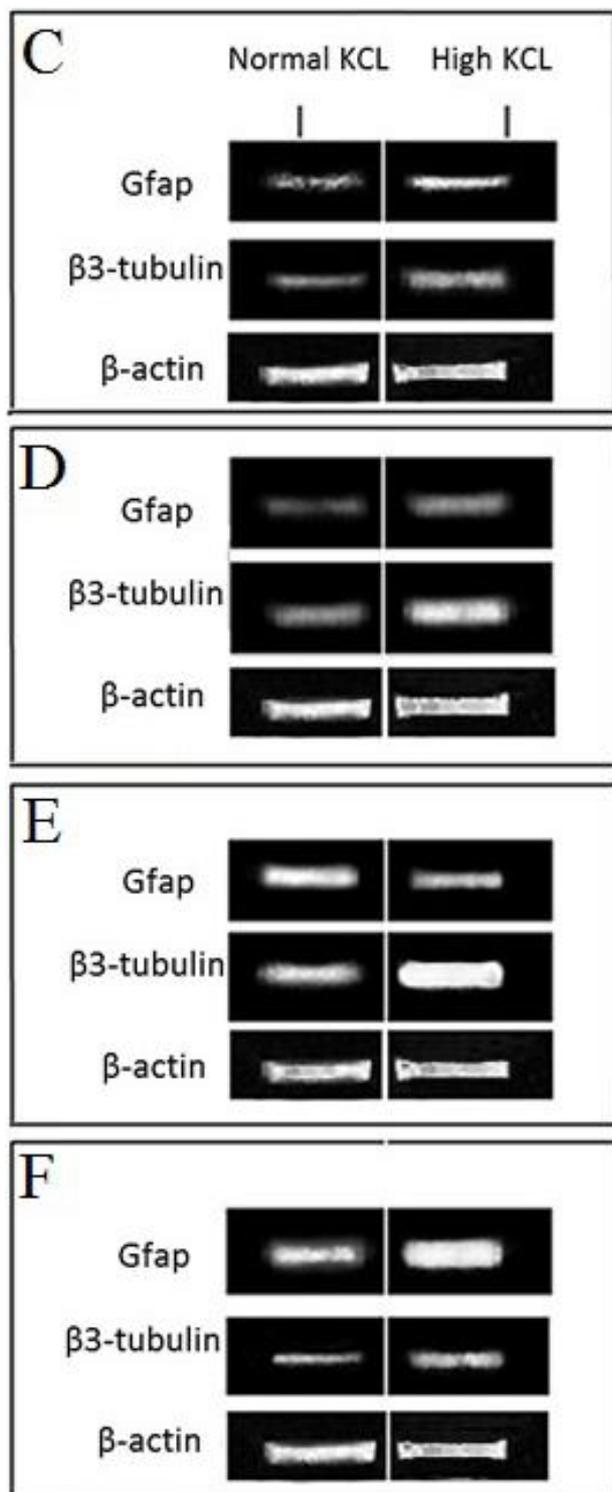


Fig. 6: sRT-PCR analysis of gene expression in NS/PCs from either normal or high KCL-treated cultures after differentiation. A, B) Semiquantitative data of the expression of neuronal (β 3-Tubulin) and astroglial (GFAP) marker mRNAs in DMEM/F12 without GFs containing either 7% FCS (control group) or 20% CSF including E-CSF (E13.5, E17) and A-CSF. Results are mean values \pm SEM from at least three independent experiments. * $P < 0.05$. C-F) Electrophoresis of PCR products using 2% agarose gel in TBE buffer. C (control), D (E13.5), E (E17), and F (Adult). β -actin is a housekeeping gene whose expression is at an identical level in all groups.

cells and PC12 cell lines, respectively. In addition, recently, *Carnicero et al.* (2013) showed the strong neurogenic and astrogligenic effects of E-CSF and A-CSF on adult neural precursor cells in mice. Other experiments have also shown the effects of A-CSF on human hipp-NS/PCs where cell S/P and neuronal and astroglial differentiations dramatically decreased and increased (Buddensiek *et al.*, 2009; Buddensiek *et al.*, 2010). We tested the same procedures for rat hipp-NS/PCs and showed that these cells were highly responsive to the developmental effects of CSF samples. It should be noted that we used this technique for NS/PCs from the hippocampus which is not in direct contact with CSF but by which these cells could be tested. We also considered a recent study in which Walker *et al.* (2008) demonstrated that the adult rodent hippocampus possesses quiescent latent NSCs which can be activated by high levels of KCL *in vitro*, resulting in the production of large neurospheres. The study was corroborant of reports regarding the reversible decrease of cell proliferation and neurogenesis in the rodent hippocampus in an age dependent manner (Gould *et al.*, 1999; Jin *et al.*, 2004; McDonald *et al.*, 2005; Montaron *et al.*, 2006; Bharathi *et al.*, 2008; Lugert *et al.*, 2010). The reason why these cells are switched off in adulthood is unclear, but one possibility is that this corresponds to a time when hippocampus growth is complete (Sun *et al.*, 2004). We tested the size of neurospheres (data not shown), compared N&H-KPC cultures and observed the neurospheres which were formed in small and large sizes as previously reported (Walker *et al.*, 2008). Corroborating Walker's report, we also tested the cells for the expression of proliferative and NS/PC markers *Ki67*, *Nestin* and *Sox2* before the differentiation process and found high levels in KCL-treated versus non-KCL-treated groups.

This elevated expression is indicative of high NSC purity. We also observed the expression of β 3-tubulin and *Gfap* in a very low level in third-passage hipp-NS/PCs from both N&H-KPC cultures. This level of expression was not significant and we noted that the expressing cells were contaminations remaining from primary cell cultures and/or those differentiated from restricted progenitors during expansion. The results also demonstrated that the third-passage hipp-NS/PCs from high KCL-treated primary cell cultures produced a large amount of differentiated cells compared to those from standard cultures demonstrating the activation of quiescent NSCs. The possibility of such activation of latent NSC enables hippocampus neuronal turnovers when necessary in specific situations. Briefly, to show the high plasticity of hipp-NS/PCs and to confirm existing quiescent NSCs, we used CSF and KCL treatments, respectively.

Considering our results, it can be concluded that the hipp-NS/PCs of adult rats are highly plastic in neuronal/astroglial differentiations. In addition, using CSF to identify NS/PC plasticity is a procedure which has not been carried out previously.

Although the results of this study could be

generalized to other rodents, further research will be needed to discover the usefulness of this method for other mammalian species, particularly the human being.

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