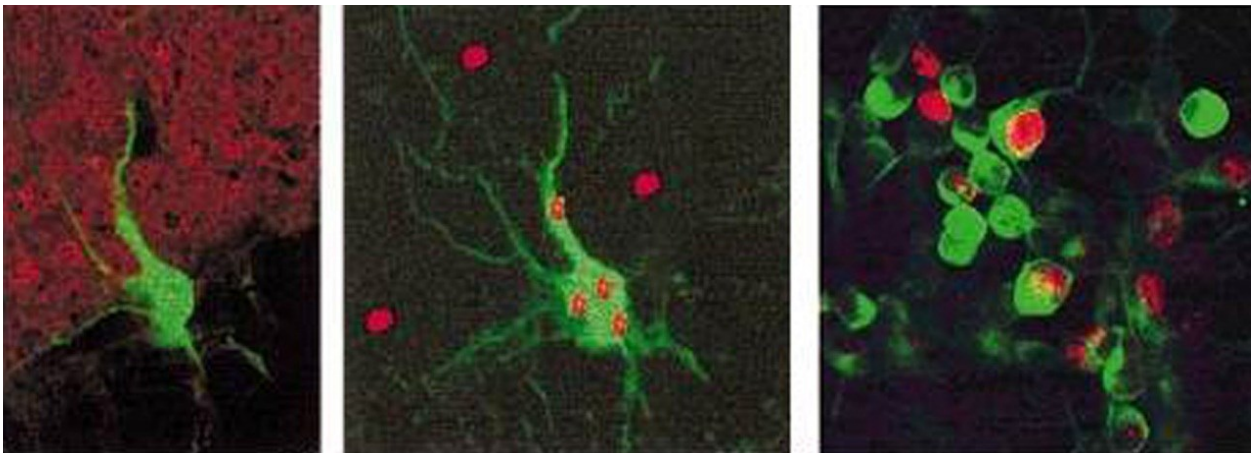

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Bangladesh Journal of Pharmacology

Research Article

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A Journal of the Bangladesh Pharmacological Society (BDPS)
Journal homepage: www.banglajol.info
Abstracted/indexed in Academic Search Complete, Agroforestry Abstracts, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index
Bangladesh J Pharmacol 2015; 10: 40-46
ISSN: 1991-0088

Retinoic acid ortho-hydroxyaniline amide promotes neurotrophin mediated cell growth and proliferation in nerve cells

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Article Info

Received: 22 November 2014
Accepted: 5 December 2014
Available Online: 11 January 2015

DOI: 10.3329/bjp.v10i1.20921

Cite this article:
Wang JP, Wang F, Wang YH, Cao PC, Wang ZG. Retinoic acid ortho-hydroxyaniline amide promotes neurotrophin mediated cell growth and proliferation in nerve cells. Bangladesh J Pharmacol. 2015; 12: 40-46.

Abstract

In the present study, effect of retinoic acid ortho-hydroxyaniline amide (RAA), an analog of retinoic acid (RA) was investigated on neurotrophin mediated cell growth and proliferation in hippocampus-derived stem cell clones. The results revealed that retinoic acid ortho-hydroxy aniline amide enhanced neuronal differentiation, increased NeuroD expression and p21 compared to proliferating cells stimulated by FGF-2. Retinoic acid ortho-hydroxyaniline amide treatment prevented decrease in RNA for trkC, enhanced expression of trkB RNA and p75NGFR. Retinoic acid ortho-hydroxyaniline amide treatment for 5 days increased the baseline expression of c-*fos* mRNA following stimulation with NGF, BDNF, and NT-3. Treatment of cultures with retinoic acid ortho-hydroxyaniline amide for 5 days followed by 5 days in serum alone increased Map2ab-positive cells compared to that of control. Therefore, retinoic acid ortho-hydroxyaniline amide is an effective agent for inducing neuronal differentiation and proliferation and is even more potent than the parent retinoic acid.

Introduction

The capacity of neural stem/progenitor cells (NSCs) to differentiate into various neural cell types can be used for the treatment of neurodegenerative diseases. However, lack of efficient strategies to control stem cell differentiation largely hinders clinical application of such NSC based cell therapies. Neural stem cells persist in regions of the adult brain where neurogenesis continues throughout life (Gage et al., 1995; Kilpatrick et al., 1995). The clones derived these cultures revealed that progenitors from the adult rat hippocampus have some of the characteristics of neural stem cells (Palmer et al., 1997). It was reported that RA or NT treatment affect molecular signals involved in initiating and promoting neuronal differentiation. The cyclins and cyclin-dependent kinases (CDKs) play a vital role in the regulation of cell-cycle (Hunter and Pines et al., 1994; Peter and Herskowitz, 1994; Sherr, 1994; Ross, 1996). The activity of cyclin-CDK is modulated by tumor supperssors (pRb and p53) (Dowdy et al., 1993; Ryan et al., 1993) or CDK inhibitors (p21, p16, and p27) (El-Deiry et al., 1995; Harper et al., 1993; Kato et al., 1994; Peter and Herskowitz, 1994; Polyak et al., 1994). CDK inhibitor, p21 correlates with nerve growth factor (NGF) during differentiation of PC12 cells (Dobashi et al., 1995; Yan and Ziff, 1995) and its up-regulation indicates changes in cell-cycle status. Two well-characterized prototypes in neuronal differentiation are neurogenin and neuroD. Both cause premature differentiation of neurons when expressed ectopically (Lee et al., 1995; Ma et al., 1996).

NTs regulate the survival and/or maturation neurons (Snider, 1994; Davies, 1994; Birling and Price, 1995; Henderson, 1996) which is consistent with higher abundance of brain-derived nerve factor (BDNF), NT-3, and their receptors, TrkB and TrkC in the developing cortex (Klein, 1994) and neural tube (Yao et al., 1994). Given the imperative role of RA and BDNF in proper

neural development and functionality, these biomolecules could be applied to facilitate controlled differentiation of stem cells to neurons for therapeutic purposes. In this work, we investigated the effects of RAA (Figure 1) an analog of RA on neurotrophin mediated cell growth and differentiation and found that RAA and NTs collaborate into the neuronal differentiation cascade by RAA.

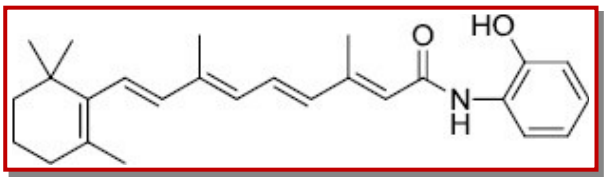

Figure 1: Structure of retinoic acid amide (RAA)

Materials and Methods

Cell culture

The clone, AP14 was obtained using previously reported protocol. Cells were plated at a density of 3 x $10^3/cm^2$ onto polyornithine/laminin-coated glass chamber slides (Nunc, Naperville, IL). Incubation of cells in N_2 supplemented medium with 20 ng/ml FGF-2 for 24 hours was followed by incubation with 0.5% fetal bovine serum (FBS) and 0.01% dimethylsulfoxide (DMSO) or 20 µM RAA in DMSO (Sigma, St. Louis, MO). For further differentiation, the medium was replaced by N_2 medium containing 0.5% FBS and one of the following: sterile water (control solvent), mouse 2.5S NGF (50 ng/mL; Boehringer Mannheim, Indianapolis, IN), rhBDNF (20 ng/mL; Genentech, South San Francisco, CA), or rhNT-3 (40 ng/mL; Genentech, South San Francisco, CA). The medium was replaced every 2 days. For inhibition of the tyrosine kinase activity of the Trk receptors, K-252a (100 nM; Calbiochem, San Diego, CA) was added to the medium.

Reverse transcription-polymerase chain reaction (PCR) analysis

Total RNA was extracted with Trizol (Invitrogen) and the density and purity detected. RNA (2 µg) of each sample was used for synthesizing cDNA through inverse transcription; 1 µL of cDNA was used to carry out PCR amplification (BioRad, Hercules, CA, USA). Primers were synthesized by Shanghai Sangon Biological Engineering Technology Company Limited. Correctness of the gene order was proved in GenBank.

RT-PCR Signal quantification

BioRad Phosphoimager and ImageQuant NT software were used for gel imaging and determination of relative intensity of each trk-specific band respectively. To normalize the absolute quantification value for RNA loading, a second set of kinetic PCR reactions were performed for the RPL27 internal control on all experimental samples. The relative abundance value was calculated for internal control as well as for trk-specific signal. The relative abundance value for each trk-specific signal was divided by the value derived from the control sequence. The normalized values for different samples were then directly compared.

Labelling and visualization of cells using bromodeoxyuridine (BrdU)

The cells after treatment with BrdU (10 mM; Sigma) were fixed with Tris-buffered 4% paraformaldehyde for 20 min. Incubation of the cells with tris-buffered 0.6% H_2O_2 for 30 min and treatment with 50% formamide/23 SSC for 2 hours was followed by 30-min incubation in 2N HCl. Neutralization in 0.1 M borate was followed by incubation with a-BrdU monoclonal antibody (1:400; Boehringer Mannheim) for 12 hours at 4°C. Incubation was then continued with horse anti-mouse secondary antibody (1: 80; Jackson Immuno-research, West Grove, PA) for 1 hour. Avidin–biotin-peroxidase system (Vectastain Elite; Vector Laboratories, Burlingame, CA) and diaminobenzidine were used for visualization of cells.

Immunofluorescence

Cells were fixed using 3.7% formaldehyde for 20 min at room temperature and then washed with PBS and wash buffer (0.1% BSA in PBS). The cells were incubated with primary antibodies for 12 hours at 4°C followed by 1 hour incubation with secondary antibodies (Jackson Immunoresearch) conjugated to fluorescein isothio-cyanate, or cyanin-3. Confocal scanning laser micros-copy (Zeiss Axiovert, Thornwood, NY; and BioRad MRC1000, Hemel Hempstead, UK) was used for visualization of labelled cells. The total number of cells was counted using nuclear counterstaining with 49,6-diamidino-2-phenylindole (DAPI; Sigma).

Statistical analysis

Statistical analyses were by analysis of variance (ANOVA) or Student 't' test. Both the average percentage and the absolute number ± standard error of the mean (S.E.M.) of immunofluorescent cells were determined by counting 10 or 50 high-power fields (203) visualized under fluorescence.

Acetylcholinesterase (AChE) staining

For AChE histochemical staining, the cells were fixed in 4% paraformaldehyde and then incubated with AChE solution for 6 days. The AChE solution was prepared by mixing 4 µM acetylthiocholine iodine, 0.2 µM ethopropazine, 2 µM copper sulfate, 10 µM glycine, and 10 mg/mL gelatin in 50 µM acetate buffer (pH 5.0). Cells were washed with water, treated with sodium sulphide and then again washed with water. The cells were suspended in silver nitrate and washed by water

for visualization of cholinergic cells.

Results

RAA enhanced neuronal differentiation by around 4-four after removal of FGF-2. Treatment of undifferentiated progenitors after FGF-2 removal with 20 µM RAA and 0.5% FBS for 5 days and then with 0.5% serum for additional 5 days lead to four fold increase in the number of Map2ab immunoreactive (IR) neurons compared to 0.5% serum alone (Table I). When undifferentiated progenitors where subjected to 10 days of RAA-treatment, 14.3 ± 1.3% adopted a neuronal lineage (TuJ1), 4.7 ± 0.5% were postmitotic neurons (Map2ab), 10.4 ± 1.8% differentiated into astrocytes (GFAP), and 1.0% ± 0.2% differentiated into oligodendrocytes (GalC).

Table I

After RAA treatment number of MAP2ab-positive neurons per square centimetre

Day	0.5% FBS	RAA
0	0.6 ± 0.4	0.6 ± 0.4
5	102 ± 13	121 ± 18
10	119 ± 21	516 ± 29
15	131 ± 19	528 ± 36

RAA; Cells treated with 20 µM RAA and 0.5% FBS for 5 days followed by an additional 5, 10, or 15 days in 0.5% FBS alone (RAA), 0.5% FBS; Cells treated with 0.5% FBS alone. The values represent the mean ± S.E.M. of three independent experiments

The results revealed that removal of FGF-2 and treatment with RAA increased NeuroD expression which remained elevated for 8 days after treatment (Figure 2A). There was significant increase in p21 on treatment with RAA compared to proliferating cells stimulated by FGF-2 (Figure 2B). The expression of p21 RNA was maximum after 4 days and was accompanied by a decrease in the number of dividing cells, indicated by BrdU incorporation (Figure 2C). Four parallel cultures treated with RAA and serum for 5 days and only serum for 5 days more were administered with BrdU for 72 hours after 0–60, 60–120, 120–180, or 180–240 hours. The cultures were evaluated on day 10 for Map2ab-IR cells incorporating BrdU (Figure 2D). We observed that 34% of the Map2ab-IR cells divided in 60 hours, <3% during 60-120 hours and <1% after 120 hours of RAA treatment.

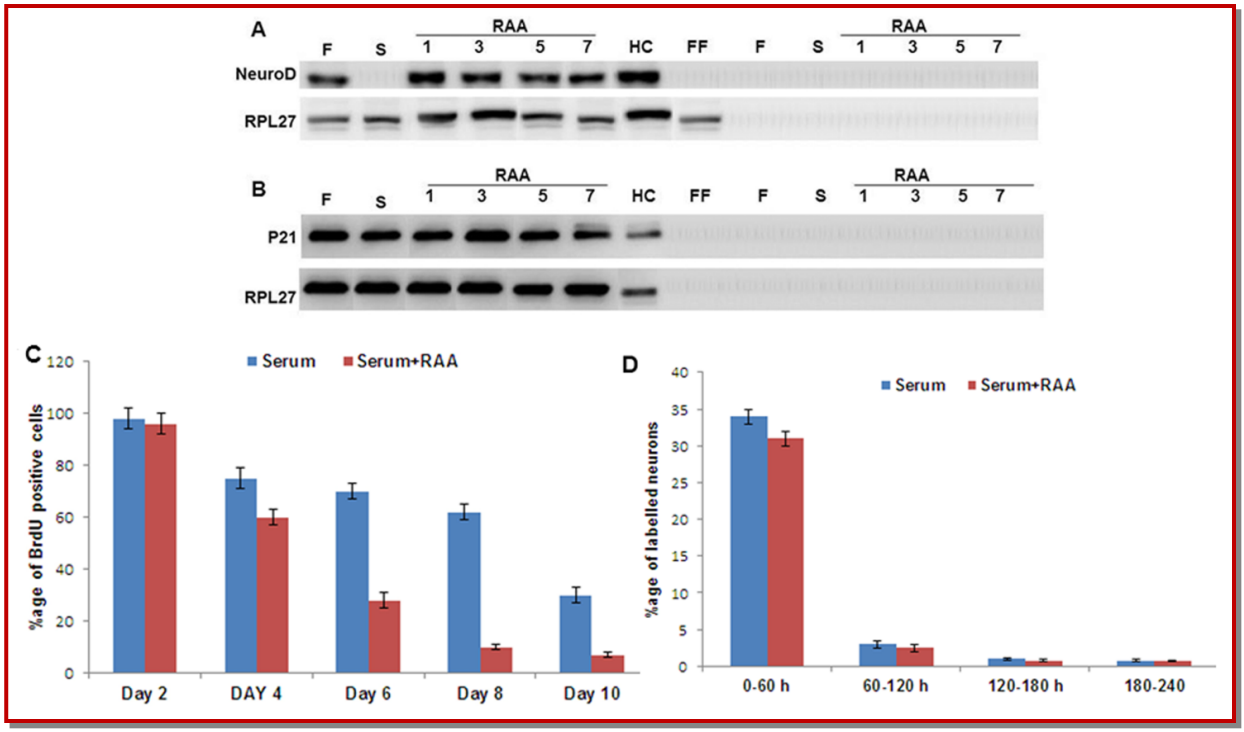

Figure 2: RT-PCR analysis of total RNA from stem cell–derived cultures treated with 20 µ*M* RA and 0.5% FBS (RAA). Control; untreated cells proliferating in the presence of FGF-2 (F), (S); cells treated with 0.5% FBS alone, or (FF); cultured Fischer 344 primary dermal fibroblasts. (A) (HC); positive control represents total RNA from an embryonic day 14 rat hippocampus. (B) (HC); positive control represents total RNA from adult rat hippocampus. The right six lanes represent control reactions without reverse transcriptase. (C) The percentage of cycling cells at various times following treatment with 20 µ*M* RA and 0.5% FBS (RAA) or 0.5% FBS alone. (D) The proportion of Map2ab-IR neurons that proliferate following RAA treatment. Values in (C–D) represent the mean ± S.E.M. of triplicate wells scored in at least three independent experiments

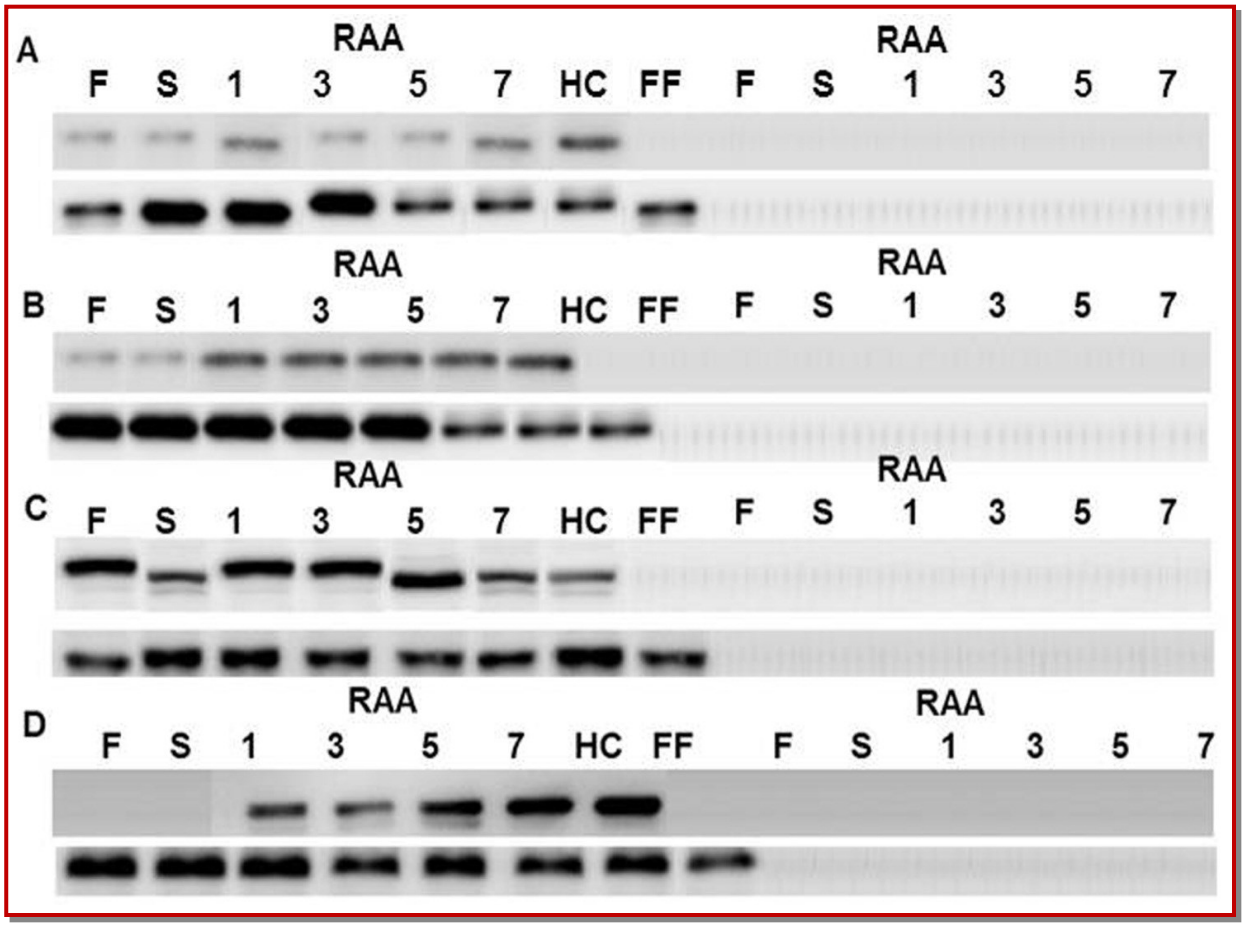

Figure 3: Regulation of trk receptor and p75NGFR expression by RAA. RT-PCR analysis of total RNA from clone AP14 treated with 20 μ*M* RA and 0.5% FBS (RAA) for 1, 3, 5, or 7 days. Controls consist of total RNA from stem cells proliferating in the presence of FGF-2 (F), stem cells treated with 0.5% FBS alone for 3 days (S), or cultured Fischer 344 primary dermal fibroblasts (FF). Positive controls consist of total RNA from PC12 cells (PC) or from adult rat hippocampal tissue (HC). The right six lanes of each panel represent control reactions without reverse transcriptase. Counts per minute (CPM) for each trk-specific band in (A–D) were normalized to the RPL27 control for each lane and then displayed as the fold change in signal relative to FGF-2-stimulated conditions

To demonstrate the effect of RAA pretreatment on NT responses, cells were first evaluated for changes in mRNA for trkA, trkB, trkC, and p75NGFR by RT-PCR analysis (Figure 3). The examination of cultures proliferating in the presence of FGF-2 revealed expression of trkA, trkB, and trkC mRNA in detectable and p75NGFR in undetectable range. However, when FGF-2 was removed and cultures were grown in serum alone there was no significant change in expression of trkA, trkB, and p75NGFR but trkC was decreased by nearly 10-fold. On the other hand, RAA treatment prevented decrease in RNA for trkC, enhanced expression of trkB RNA by two-fold and that of p75NGFR by more than 7 fold compared to proliferating FGF-2-stimulated cultures. RNA for trkA seemed to be relatively unaffected by RAA, though we noted RAA-independent inter sample variability (Figure 3A-D).

Treatment of proliferating controls with NTs induced minimal c-*fos* mRNA. Treatment with 0.5% FBS alone provided a subtle increase in c-*fos* response to all three NTs, and pre-treatment with RAA for 5 days increased the baseline expression of c-*fos* mRNA following stimulation with NGF, BDNF, and NT-3. Pretreatment with K-252a, a selective inhibitor of Trk receptor tyrosine kinase (Berg et al., 1992), blocked c-*fos* induction by each NT, confirming signal transduction via receptor tyrosine kinase signalling.

We observed a small number of c-*fos*-positive cells in proliferating cultures (Figure 4A) and treatment with 0.5% FBS alone lead to a little increase in number of cells responding to a 2-hours exposure to each NT. However, the number of cells responding to all three NTs was significantly enhanced after 5 days of RAA treatment.

Examination of phenotype in c-*fos*-positive cells revealed that most cells were undifferentiated (Map2abc), consistent with the minimal differentiation

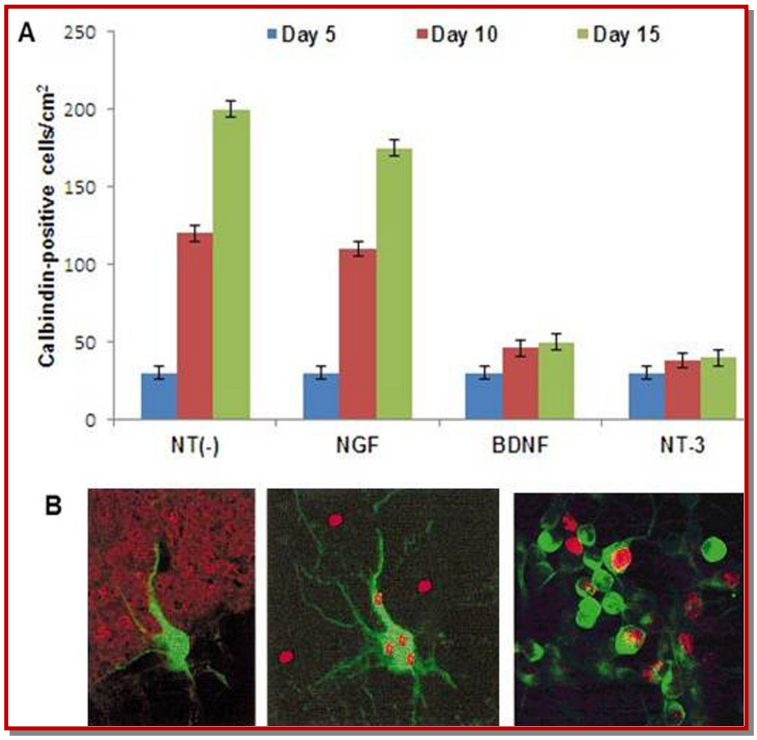

Figure 4: Immunofluorescent detection of c-*fos*-immunoreactive cells after NT stimulation. (A) Cells were cultured in the presence of FGF-2 (FGF-2), 0.5% FBS alone (0.5% FBS), or 20 µ*M* RAA and 0.5% FBS (RAA) for 5 days. Then stimulated for 2 hours with: vehicle alone Control (C); 50 ng/ml NGF (N); 20 ng/ml BDNF (B); or 40 ng/ml NT-3 (3) separately or in combination. Approximately 1600 total cells were scored for FGF-2, 5700 cells for 0.5% FBS, and 3000 cells for RAA. Values represent the mean ± S.E.M. of triplicate wells scored for each of four independent experiments. (B) Colocalization of c-*fos* (red) with MAP2abc (green, a), MAP2ab (green, b), and p75NGFR (green, c). AP14 cells were treated with 20 µ*M* RAA and 0.5% FBS (RAA) for 5 days and stimulated

expected at this early time point (Figure 4B). However, when the ratio of responding cells was evaluated in undifferentiated cells versus Map2ab-positive neurons, 3% (11 of 400) of the undifferentiated cells were c-*fos* positive, whereas 47% (186 of 400) of the Map2ab-positive neurons were c-*fos* positive, suggesting that those cells differentiating into neurons were much more responsive as a population than the undifferentiated cells. Interestingly, p75NGFR-positive cells were abundant but very few were also c-*fos* positive, suggesting that p75NGFR played little if any role in signaling by these three NTs in stem cell-derived progenitors.

After cells were cultured with RAA and serum for 5 days, the medium was replaced by RAA free medium containing NGF, BDNF, or NT-3 for an additional 5 or 10 days. Treatment of cultures with RAA for 5 days followed by RAA free medium for 5 days increased Map2ab-positive cells over 4 times compared to that of control. Treatment of cultures with NT alone had negligible effect on number of total neurons (Figure 5A), however, NT treatment exhibited a significant effect on number of neurons expressing GABA, AChE, TH, or calbindin (Figure 5B-D). BDNF and NT-3 but not NGF increased the GABA (most abundantly encountered phenotype) and number of TH-positive and calbindin-positive cells. The number of AChE-positive cells was increased by BDNF.

Discussion

The results demonstrated that retinoic acid amide significantly enhanced differentiation of stem cell cultures by promoting the response to neurotrophic factors. Although NTs and retinoic acid amide treatment did not affect the number of neurons but facilitated neurons to attain mature phenotype. The cultures generating only GABA-positive neurons on treatment with retinoic acid amide and NTs could generate dopaminergic (TH) and cholinergic (AChE) phenotypes. Retinoic acid amide treatment facilitated rapid induction of NeuroD and up-regulation of the cell cycle regulatory protein, p21. Treatment of neural cell cultures with retinoic acid amide lead to half of the neurons with a GABA phenotype in response to BDNF or NT-3 and smaller proportions were immunoreactive for TH or AchE.

In response to all the three NTs, c-*fos* was induced and three Trk receptors were co-expressed in almost all the cells. Retinoic acid amide leads to a minor enhancement in c-*fos* mRNA expression in in absence of NT stimulation. The direct induction of c-*fos* expression by retinoic acid amide may be to paracrine and/or autocrine effects. RT -PCR analysis after 5 days of RA treatment revealed marginally detectable amounts of NGF mRNA, but not BDNF or NT-3 mRNA. This low level of NGF may not have been sufficient for maximal stimulation of c-*fos* (exogenous NGF increased c-*fos* responses), but it may have been sufficient to generate maximal effects on acquisition of the phenotypes evaluated here.

Conclusion

The collective process comprising of FGF-2 signalling, RAA-induced changes in gene expression, and activation of NT-dependent signal cascades cause increase in phenotype plasticity of neurons. Therefore, a variety of neuronal types arise from a single adult hippocampus-derived stem cell.

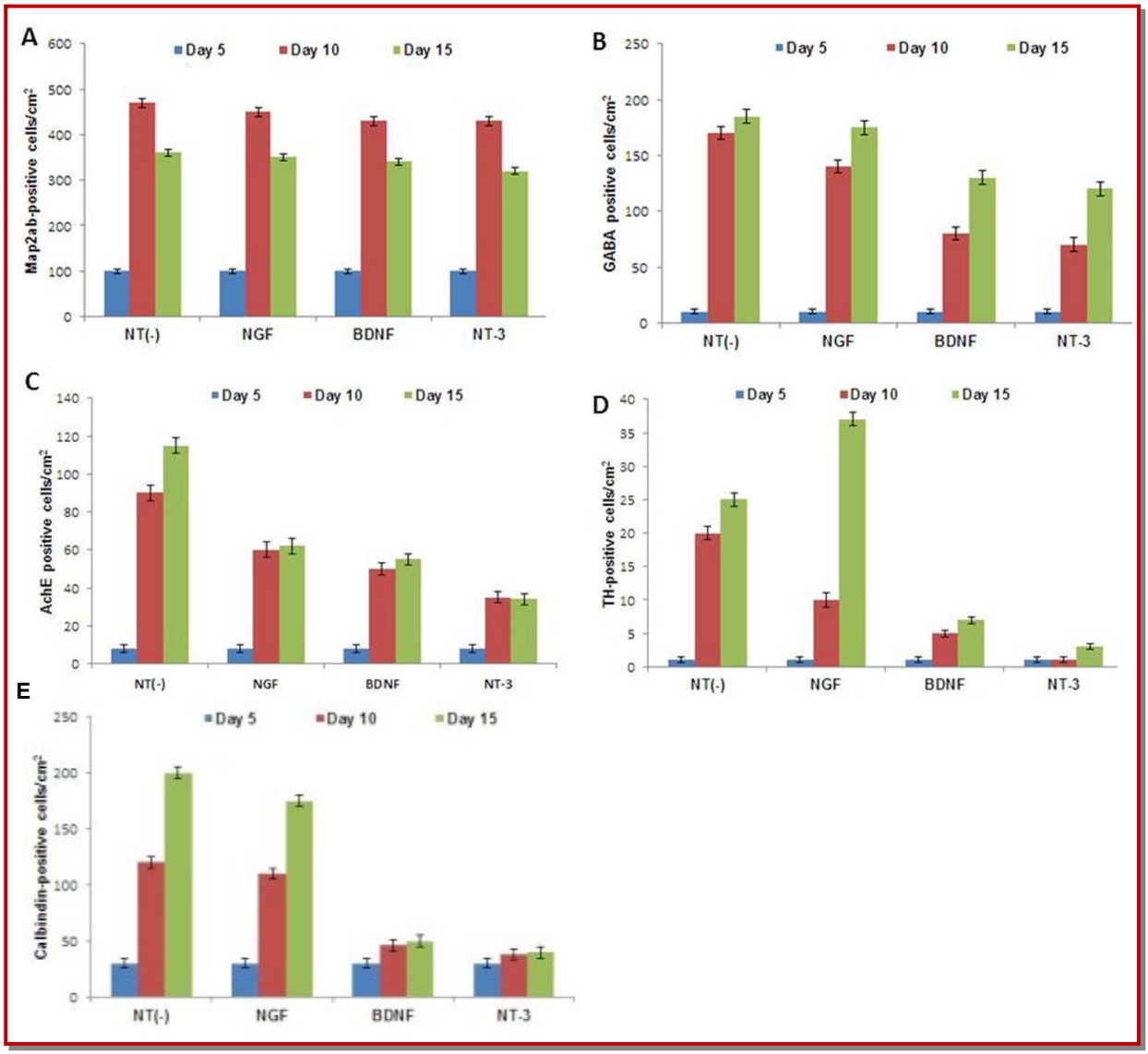

Figure 5: Effect of RAA followed by NTs on neuronal maturation. (A) Density of MAP2ab-positive cells in AP14 stem cell cultures on day 5, 10, and 15 following treatment with the indicated neurotrophic factors. Approximately 15,000 cells were scored on day 5, 10,000 cells on day 10, and 8000 cells on day 15. Values represent the mean ± S.E.M. of four independent experiments. (B-E) The density of cells positive for GABA, AChE, TH, and calbindin. Cells were treated with 20 µM RAA and 0.5% FBS (RAA) for 5 days and with the indicated NTs for another 5 or 10 days. Values represent the mean ± S.E.M. of triplicate wells scored in at least three independent experiments

Financial Support

Self-funded

Conflict of Interest

Authors declare no conflict of interest

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