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Synergistic Effect of Schwann Cells and Retinoic Acid on Differentiation and Synaptogenesis of Hippocampal Neural Stem Cells *in vitro*[1]

XUE-BAO ZHANG, YUAN-SHAN ZENG[2], WEI ZHANG, YA-YUN CHEN, WEI ZHANG, YI XIONG, AND SUI-JUN CHEN

Division of Neuroscience, Department of Histology and Embryology, Zhongshan Medical College, Sun Yat-Sen University, Guangzhou 510080, Guangdong, China

Objective To investigate the synergistic effect of Schwann cells (SCs) and retinoic acid (RA) on differentiation and synaptogenesis of neural stem cells (NSCs) derived from hippocampus of neonatal rats. **Methods** The classical method for 2×2 factorial analysis experiment was used to assess synergistic action of SCs and RA. NSCs were treated with RA, SCs, and SCs + RA in DMEM/F12 with 0.5% fetal bovine serum for six days, respectively. Double immunofluorescent staining was used to detect the differentiation of NSCs including nestin, glial fibrillary acidic protein (GFAP) and Map2. The expression of PSD95 was used to demonstrate synaptogenesis. **Results** After NSCs were treated with RA or SCs, the expression of nestin and GFAP was significantly decreased while the expression of Map2 and PSD95 was significantly increased in comparison with the control. Factorial ANOVA showed that interactions between SCs and RA could induce the expression of Map2 and PSD95. **Conclusion** SCs and RA could promote synergistically the neuronal differentiation and synaptogenesis of hippocampal neural stem cells *in vitro* while they decreased the astrocytes and nestin positive NSCs.

Key words: Neural stem cell; Neuronal differentiation; PSD95; Schwann cells; Retinoic acid

INTRODUCTION

Schwann cells (SCs) secrete growth factors or NGF-like proteins, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3, basic fibroblast growth factor (bFGF), and glial cell line-derived neurotrophic factor (GDNF)[1-3] . These factors can enhance the survival of neural stem cells and promote their differentiation into neurons[4-5]. It has been shown that SCs can promote the survival and differentiation of co-cultured neural stem cells *in vitro*[6-7] and play an important role in neuron-to-neuron communication[8]. All-*trans* retinoic acid (RA), a member of vitamin A derivatives, is known to profoundly influence cell differentiation, survival, and lineage decision making. RA enhances the production of neurons from a mitotically active population of neural crest cells[9]. An accompanying effect of RA is to sustain or up-regulate trkA, trkB, trkC, and p75NGFR expression. Without RA treatment, cells are minimally responsive to neurotrophins (NTs)[10]. On the basis of these observations, the present study tested the hypothesis that neonatal rat SCs and RA could synergistically affect the neuronal differentiation and synaptogenesis of neonatal rat neural stem cells (NSCs).

MATERIALS AND METHODS

Culture of Neonatal Rat NSCs

Neural stem cells were isolated from hippocampus of neonatal Sprague-Dawley (SD) rat (3-5 days). Briefly, the hippocampus tissue was collected in cold Hank's solution and mechanically excised under a dissection microscope, cells obtained by mechanical dissociation were centrifuged at 1000 rpm for 5 min. The precipitation was resuspended by gentle trituration with a fire-polished Pasteur pipette to dissociate into single cells and allocated averagely into flasks containing a defined serum-free culture medium. The medium was a mixture (1:1) of Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12; GIBCO), containing 100 units/mL penicillin, 100 μg/mL streptomycin, 20 μL/mL B27

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[2]Correspondence should be addressed to Yuan-Shan ZENG. Tel: +86-20-87331452. E-mail: yzeng@sums.edu.cn

Biographical note of the first author: Xue-Bao ZHANG, female, born in 1977, Ph. D., majoring in spinal cord injury and repairment. E-mail: sxzxb@163.com

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(GIBCO, Grand Island, NY) and 20 ng/mL recombinant human basic fibroblast growth factor (bFGF, GIBCO), and incubated for 3-5 days at 37℃ with 5% CO_2/95% air. The experiments were approved by the Animal Care and Use Committee of the Sun Yat-Sen University.

Culture of Neonatal Schwann Cells

SC cultures were prepared from bilateral sciatic and brachial nerves of 3-5-day-old SD rats according to the established procedure[11] with minor revision. Briefly, after dissection, the epineurium and connective tissue were removed under a microscope, the sciatic and brachial nerves were cut into 2 mm^3 fragments and treated with 0.16% collagenase for 20 min at 37℃, then centrifuged at 1000 rpm/min for 5 min. The supernatant was removed, the cells were resuspended in DMEM/F12 containing 10% fetal bovine serum, 2 μmol/L forskolin (Sigma) and 20 μg/mL bovine pituitary extract (Sigma). The cells were seeded in flasks coated with poly-lysine and incubated at 37℃ with 5% CO_2/95% air. The medium was changed twice a week.

Experimental Grouping

For *in vitro* differentiation studies, a poly-L-lysine -coated 24-well plate was used. According to different treatment factors, NSCs were divided into 4 groups, with each group in six wells. SCs were seeded into SC group and SCs+RA group. When they were in exponential growing state, SCs were washed 3 times with Hank's solution and then the medium was replaced by DMEM/F12 supplemented with 0.5% FBS and 20 μL/mL B27. Hoechst 33342 (Sigma) was added into the medium of NSCs at the final concentration of 10 μg/mL after subcultured for 2 weeks. Two hours later, NSCs were washed with Hank's solution and equally divided into 4 groups respectively with a differentiation medium containing 0.5% fetal bovine serum instead of bFGF and then 0.5 μmol/L RA in dimethylsulfoxide (DMSO) was added into RA group and RA +SCs group at dark. DMSO (0.01%) was added into control group as control solvent. The medium was changed every other day.

Immunofluorescence

All staining procedures were carried out at room temperature otherwise indicated. Phosphate-buffered saline (PBS) (0.01 mol/L) was used in all washing steps. Cells were rinsed 3 times with PBS 6 days after culture, then fixed with 4% paraformaldehyde in PBS for 10 min. Primary antibodies were diluted in 0.01 mol/L PBS containing 1% bovine serum albumin and 0.3% Triton X-100. All primary antibodies were diluted to their optimum concentration: monoclonal anti-nestin (1:1000, Sigma), monoclonal anti-Map2 (Sigma, 1:500), monoclonal anti-PSD95 (1:500, BD Bioscience), polyclonal anti-glial fibrillary acidic protein (GFAP, 1:500, Boster). After washing, normal goat serum was used for 30 min to prevent non-specific conjugate binding. Then the cells were incubated overnight at 4℃ with polyclonal or monoclonal antibodies. After washed with PBS, an indirect immunofluorescence was performed, using biotinylated goat anti-mouse IgG and biotinylated goat anti-rabbit IgG at 1:100 dilution for 30 min followed by incubation in SABC-cy3 (SABC Kit, Boster) for 30 min. Negative control sections receiving the same treatment were incubated in PBS. The labeled cells were visualized under a fluorescent optical microscope.

Cell Counts

Three samples of each culture were examined. For each treatment, five randomly selected high-power fields (20×) were counted for neural progenitors ($nestin^+$), neurons ($Map2^+$), and astrocytes ($GFAP^+$) visualized under a fluorescent optical microscope, the cell numbers were expressed as a percentage of the total number of NSCs assessed by hoechast 33342 staining.

Statistical Analysis

The data were expressed as $\bar{x} \pm s$ and analyzed using factorial design ANOVA. All the statistical analyses were performed using SPSS 10.0 software. The significance level was set at 0.05. The 2×2 factorial analysis experiment was used. Factorial design ANOVA was employed to clarify the interactions between SCs and RA. The interaction was considered as additive if $P>0.05$, and synergistic or antagonistic when $P<0.05$.

RESULTS

Hippocampus-derived cells in culture proliferated and formed floating neurospheres. Immunohistochemical analysis of neurospheres from culture revealed that NSC specific protein nestin was found in 95% of cells. In the differentiation medium, NSCs attached to the dish 24 h later, many of which prolonged cellular processes. To assess the types of cells present in culture after 6 days, the total number of progenitors ($nestin^+$), neurons ($Map2^+$), and astrocytes ($GFAP^+$) was counted respectively in culture after treatment with RA, SCs and SCs+RA. In the absence of treatment, a small number of cells expressed neuronal antigens. Addition of RA, SCs or SCs+RA increased

the number of neurons (Fig. 1) and decreased the number of progenitors (Fig. 2) and astrocytes (Fig. 3). To assess the maturation of neurons differentiated from NSCs, the expression of PSD95 was tested to reflect synaptogenesis of neurons. The results showed that few neurons expressed PSD95 in the absence of treatment and even after treatment with RA. However, addition of SCs or SCs+RA increased the number of neurons expressing PSD95 (Fig. 4).

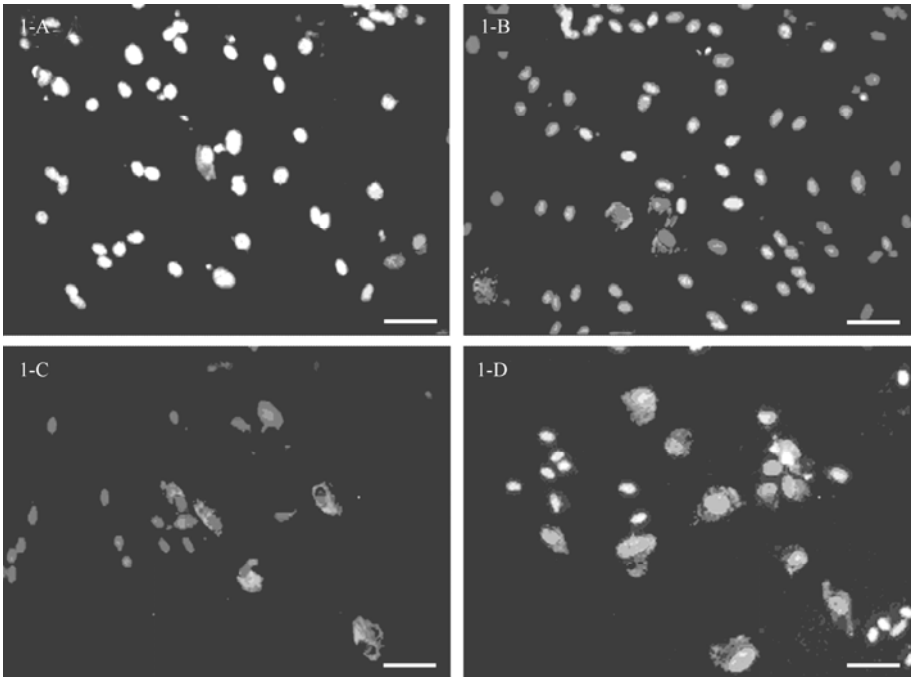

FIG. 1. Double immunofluorescentstaining of Map2 positive cells differentiatied from NSCs. Addition of RA, SCs, or SCs+RA increased the number of neurons (1-B, 1-C, 1-D) compared with control (1-A). The nuclei was labelled blue by Hoechst 33342 while cellular body was positively stained (red). Scale bar =10 μm.

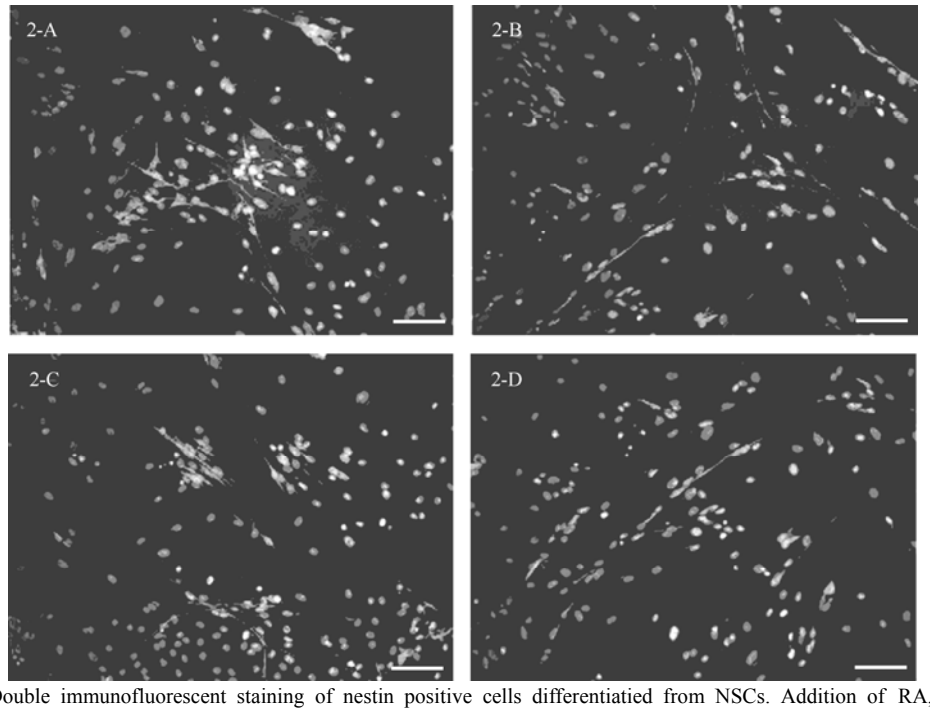

FIG. 2. Double immunofluorescent staining of nestin positive cells differentiatied from NSCs. Addition of RA, SCs, or SCs+RA decreased the number of progenitors (2-B, 2-C, 2-D) compared with control (2-A). The nuclei was labelled blue by Hoechst 33342 while cellular body and processes were positively stained (red). Scale bar =20 μm.

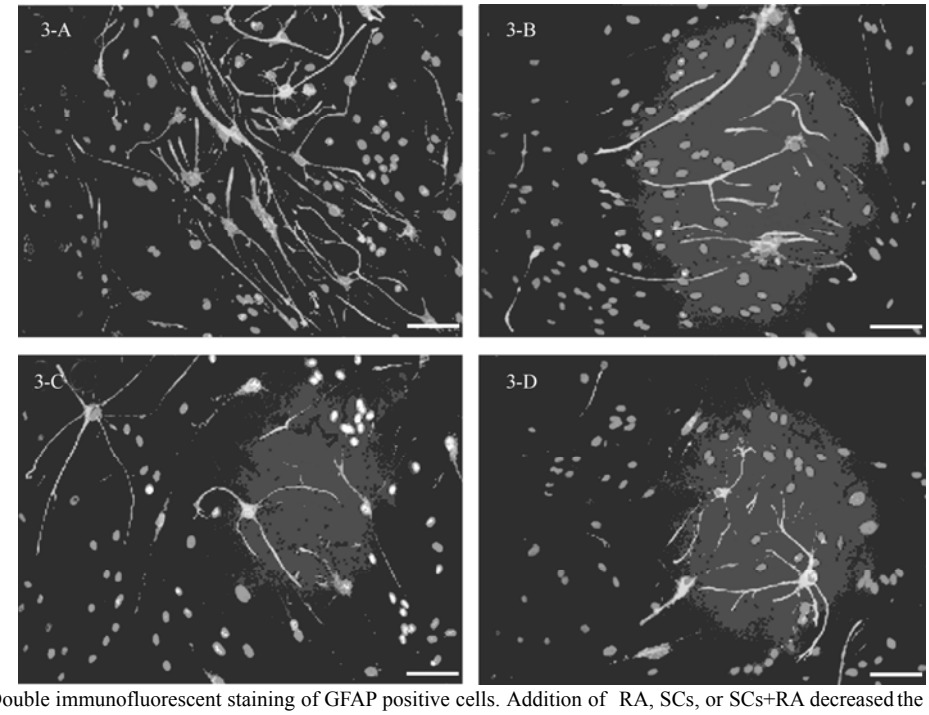

FIG. 3. Double immunofluorescent staining of GFAP positive cells. Addition of RA, SCs, or SCs+RA decreased the number of astrocytes (3-B, 3-C, 3-D) compared with control (3-A). The nuclei was labelled blue by Hoechst 33342 while cellular body and processes were positively stained (red). Scale bar =20 μm.

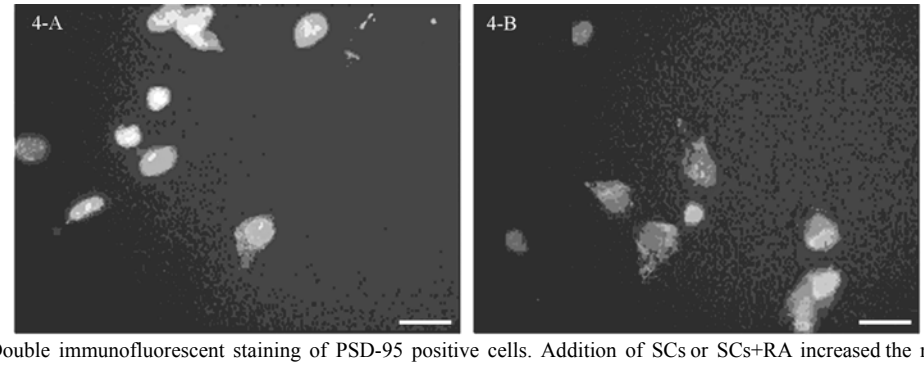

FIG. 4. Double immunofluorescent staining of PSD-95 positive cells. Addition of SCs or SCs+RA increased the number of PSD-95 positive cells (4-A, 4-B). The nuclei was labelled blue by Hoechst 33342 while cellular body were positively stained (red). Scale bar =5 μm.

When NSCs were treated with RA, SCs, and SCs+RA, the expression of Map2 was significantly increased while the expression of nestin was significantly decreased. GFAP was seen in comparison with the controls (P<0.01). The expression of PSD95 was significantly increased in NSCs treated with SCs and SCs+RA (P<0.01) and no obvious increase was found in the group treated with RA. Factorial analysis showed that interactions between SCs and RA could induce the expression of Map2 and PSD95 (P<0.01) while interaction between SCs and RA could not decrease the expression of nestin and GFAP (Table 1).

DISCUSSION

The development of the nervous system is a complicated, multi-step process. From neurogenesis to synaptogenesis, individual neurons encounter a variety of environmental factors that can profoundly influence their growth, differentiation, and survival[12]. Neural stem cells are expected to be useful in cellular therapies for neurological trauma and diseases. Therefore, it is important to study the controlled differentiation of NSCs into neurons. Researchers who alter the differentiation properties of neural progenitors may bias these cells to acquire or maintain

TABLE 1

Effects of Retinoic Acid (RA) and Schwann Cells (SCs) on the Differentiation and Neuronal Maturation of Neural Stem Cells (NSCs) Cultured 6 Days *in vitro* ($\bar{x} \pm s$)

Groups	Factors		Map2 Positive Cells (%)	Nestin Positive Cells (%)	GFAP Positive Cells (%)	PSD95 Positive Cells (%)
Control	0	0	5.67±0.71	19.38±2.20	59.05±2.06	0.22±0.23
RA	RA	0	14.75±1.47[b]	15.43±1.84[b]	47.24±1.59[b]	0.37±0.36
SCs	0	SCs	25.67±2.71[b]	12.92±0.78[b]	33.79±1.72[b]	6.96±1.76[b]
SCs+RA	RA	SCs	47.54±2.21[a,b]	9.64±2.34[b]	20.47±1.88[b]	19.76±1.92[a,b]

Note. [a]$P<0.01$, factorial analysis of interaction effect between SCs and RA. [b]$P<0.01$, compared with the control group.

desired neuronal phenotypes *in vitro* or *in vivo*. So the characterization of appropriate culture conditions and regulators of differentiation is necessary to convert progenitors into neurons that can integrate into functional neural networks[13-14].

One of the hallmarks of mature CNS neurons is their ability to form synapses[15]. The expression of PSD95 is correlated with synaptogenesis. Thus PSD95 can be used as a reliable marker of nerve terminal differentiation.

Our results indicated that treatment with RA alone could significantly induce neuronal differentiation and decrease astroglial differentiation and progenitors of NSCs, but it cannot promote synaptogenesis. All-*trans* retinoic acid receptors are members of the steroid receptor superfamily.There is evidence that mRNA isolated from the embryonic mouse striatum and the developing nervous system is a major target for RA actions[9,16]. It is suggested that RA might induce a retinoid-specific effect on stem cell differentiation. In our study, although RA induced part of NSCs to differentiate into neurons, the differentiation rate was very low (14.75%±1.47%) and the neurons were premature for few PSD-95 positive cells.

SCs are known to play an importmant role in the regeneration of mammalian peripheral nerves[17]. Their effect may depend on the production of neurotrophic and/or supportives factors[18]. Recently, it has been shown that SCs induce neuronal differentiation of NSCs and bone marrow stromal cells, but there is no evidence that SCs induce synaptogenesis[7,19]. However, it was reported that trophic stimulation maintains spinal neurons in the growth state, and Schwann cell-derived factors allow them to switch to the synaptogenic state[12-13]. In our experiment, treatment with SCs alone promoted neuronal differentiation, synaptogenesis and decreased astroglial differentiation, progenitors of NSCs, but the rate of synaptogenesis was not very high. So further work is deemed necessary to optimize and improve the effectiveness of SCs.

RA-dependent upregulation of neurotrophin receptors has been shown in the developing nervous system[20]. There is evidence that FGF-2 responsive rat NSCs spontaneously express trkC and lower amounts of trkA or trkB[10]. After treatment with RA, the cells can immediately respond to all three neurotrophins. RA could also maintain or increase the amount of trkA, B, and C, and p75NGFR mRNA. This is consistent with several papers recently published, showing that RA plays a role in acquisition of responsiveness to NTs. Since SCs secretes several neurotrophic factors including NGF, BDNF, and NT-3, they may play a limited role in promoting neuronal differentiation in the absence of RA stimulation[10], suggesting that SCs be more effective if the cells are treated with RA. An interesting finding in this study was that neonatal rat SCs and RA had a synergistic effect on the neuronal differentiation and synaptogenesis of neonatal rat NSCs. In contrast, the decreasing effect of SCs and RA on astrocytes and nestin postive NSCs are additive. To our best knowledge, the present study is the first report on such findings. Johann *et al*.[21] found that all RA receptors and retinoid X receptors were expressed in Schwann cell primary cultures, and treatment with RA could change the concentration of ciliary neurotrophic factor (CNTF) mRAN. It is suggested that the synergistic effect of SCs and RA might have two different paths: either RA directly acts on SCs or SCs and RA directly act on NSCs respectively to affect the neuronal differentiation of NSCs.

In summary, the present results confirmed that retinoic acid and Schwann cells played a synergistic role in promoting the neuronal differentiation and synaptogenesis of hippocampal neural stem cells *in vitro*. The effect that SCs and RA decreased the astrocytes and nestin postive NSCs were additive.

The future work should be undertaken to explore the underlying mechanism.

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