The role and mechanism of action of activin A in neurite outgrowth of chicken embryonic dorsal root ganglia

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Summary

Activin A, a member of the transforming growth factor β (TGFβ) superfamily, plays an essential role in neuron survival as a neurotrophic and neuroprotective factor in the central nervous system. However, the effects and mechanisms of action of activin A on the neurite outgrowth of dorsal root ganglia (DRG) remain unclear. In the present study, we found that activin A is expressed in DRG collected from chicken embryos on embryonic day 8 (E8). Moreover, activin A induced neurite outgrowth of the primary cultured DRG and maintained the survival of monolayer-cultured DRG neurons throughout the observation period of ten days. Follistatin (FS), an activin-binding protein, significantly inhibited activin A-induced neurite outgrowth of DRG, but failed to influence the effect of nerve growth factor (NGF) on DRG neurite outgrowth. Furthermore, the results showed that activin A significantly upregulated mRNA expression of activin receptor type IIA (ActRIIA) and calcitonin gene-related peptide (CGRP) in DRG, and stimulated serotonin (5-HT) production from DRG, indicating that activin A might induce DRG neurite outgrowth by promoting CGRP expression and stimulating 5-HT release. These data suggest that activin A plays an important role in the development of DRG in an autocrine or paracrine manner.

Key words: Activin, Follistatin, Neurite outgrowth, Dorsal root ganglia

Introduction

Activin A, a member of the transforming growth factor β (TGFβ) superfamily, is a multifunctional growth and differentiation factor (Liu et al., 2006; Kingsley, 1994). It has a broad range of physiological activities, including regulation of secretion of follicle-stimulating hormone (FSH) and follistatin (FS) from the anterior pituitary (Liu et al., 1996), spermatogenesis (Mather et al., 1990), stimulation of erythroblast differentiation (Yu et al., 1987), embryonic development (Thomsen et al., 1990) and proliferation of avian auditory sensory epithelium (McCullar et al., 2010). As a neurotrophic and neuroprotective factor, activin A is also involved in maintaining survival of central nervous system (CNS) neurons in culture and protecting neurons from neurotoxic injury (Iwahori et al., 1997; Schubert et al., 1990; Suzuki et al., 2010; Wu et al., 1999). Previous studies revealed that expression of various kinds of growth and differentiation factors were upregulated after brain injury, including an increase in activin A mRNA expression (Hughes et al., 1999a; Lai et al., 1996; MacConell et al., 1996; Schneider et al., 2000). However, it has not yet been determined whether activin A might stimulate neurite outgrowth of dorsal root ganglia (DRG) in the peripheral nervous system.

The neuropeptides calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP) and their receptors are widely expressed in the nervous systems (Cochaud et al., 2010; Wimalawansa, 1996). Previous studies have reported that CGRP expression in surviving neurons within damaged regions of the hippocampus is likely to be an important, and possibly protective, component of the response of the nervous system to injury (Bulloch et al., 1998). As a neurotransmitter and neuromodulator, serotonin (5-HT) influences neurite outgrowth and synaptogenesis in the nervous systems of several species (Saudou and Hen, 1994; Zhou and Cohan, 2001).

In this study, we focused on the presence of activin A in DRG and its effect on neurite outgrowth of DRG from chicken embryos on embryonic day 8 (E8). In addition, expression of type II receptor of activin (ActRII), neuropeptide expression and 5-HT release were analyzed in order to define the possible mechanism of activin A action.

Results

Activin A and its receptors are expressed in the DRG of chicken embryos

Activin A expression and action have been reported in the central nervous system and expression of its receptors has been observed in the peripheral nervous system (Kos et al., 2001). We therefore sought to determine whether activin A itself is expressed in the peripheral nervous system. Reverse transcription polymerase chain reaction (RT-PCR) revealed expression of not only type IIA and IIB receptors of activin (ActRIIA and ActRIIB), but also activin A mRNA in chicken DRG (Fig. 1A). Furthermore, mature activin A protein expression was also detectable in DRG by immunohistochemical staining (Fig. 1B). These data suggest that activin A might play an important role in DRG in an autocrine or paracrine manner.

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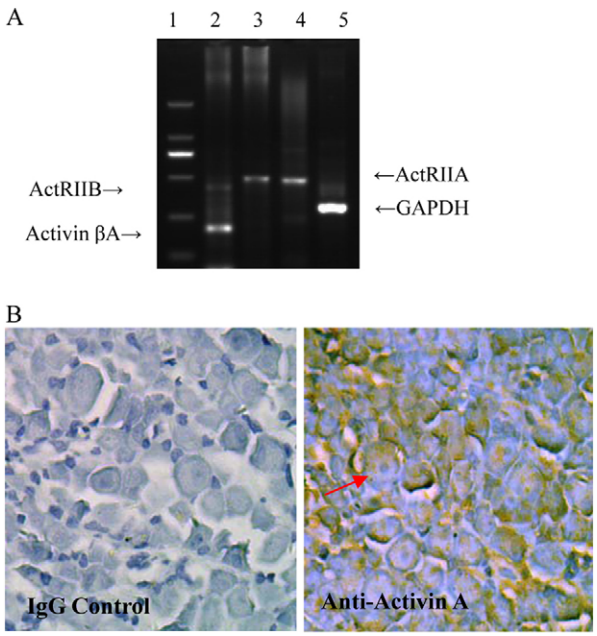


Fig. 1. Activin βA and its receptor expression in chicken DRG. (**A**) Activin βA, ActRIIA and ActRIIB mRNA expression was examined by RT-PCR. Lane 1, molecular weight marker; Lane 2, activin βA; Lane 3, ActRIIB; Lane 4, ActRIIA; Lane 5, GAPDH. (**B**) Activin A protein expression was analyzed by immunohistochemical staining. For IgG control staining, sections were incubated with normal rabbit IgG instead of anti-activin A antibody. Red arrow indicates immunostained cells for activin A. Magnification: 200×.

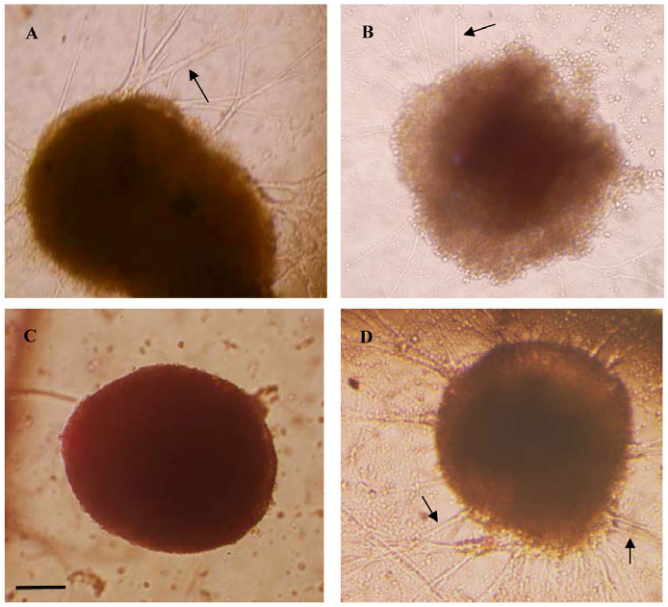


Fig. 2. FCS-dependent neurite outgrowth of cultured DRG of chicken embryos. (**A**) Significant neurite outgrowth was observed from DRG cultured with 10% FCS-DMEM at 37℃ with 5% CO_2 for 3 days. (**B**) Some neurites grew from DRG incubated with 2.5% FCS-DMEM. (**C**) Neurite outgrowth was not observed in DRG maintained with 0.5% FCS-DMEM. (**D**) Significant neurite outgrowth was observed in the positive control DRG incubated in 0.5% FCS-DMEM containing 4 ng/ml NGF for 3 days. Arrows indicate neurites. Scale bar: 50 μm.

FCS-dependent DRG neurite grows

The results above provide strong evidence that activin A and its receptors are present on embryonic DRG neurons. We therefore sought to determine the role of activin A in the peripheral nervous system by culturing primary DRG and observing neurite outgrowth. We observed neurite outgrowth from DRG cultured in either 10% FCS-DMEM or 2.5% FCS-DMEM for 3 days (Fig. 2A,B). However, no neurite extension was found in DRG cultured in 0.5%FCS-DMEM (Fig. 2C), suggesting that neurite growth requires FCS. NGF is one of the target-derived neurotrophic factors and is responsible for the survival and maintenance of specific subsets of peripheral neurons. As NGF can promote DRG neurogenesis and neurite growth (Levi-Montalcini et al., 1996, Kashiba et al., 1998; Ma et al., 1999; Rifkin et al., 2000), it was used in our present study as a positive control. Although neurite outgrowth was undetectable in DRG cultured in 0.5% FCS-DMEM, we did observe obvious neurite outgrowth from these DRG when NGF was added to the culture (Fig. 2D). Therefore, 0.5% FCS-DMEM was chosen to examine neurite outgrowth of embryonic DRG in subsequent experiments.

Activin A stimulates neurite outgrowth of DRG

We tested the effect of activin A using the culture system described above. Although no neurite extension could be observed in DRG cultured in 0.5% FCS-DMEM (Fig 3A–C), we noted obvious neurite outgrowth in DRG cultured with 5 ng/ml activin A (Fig. 3D–F) or 4 ng/ml NGF (Fig. 3G–I). To confirm activin A-induced DRG neurite outgrowth, DRG were cultured in serum-free medium. We found that no neurites grew in DRG cultured in serum-free medium for 3 days, but 4 ng/ml NGF- and 5 ng/ml activin A-treated groups showed the thin neurite outgrowth characteristic of chicken embryonic DRG (Fig. 3J–L). These results suggest that activin A has a similar role to NGF in the promotion of neurite outgrowth.

Follistatin blocks activin A-induced neurite outgrowth of DRG

To confirm that activin A promotes neurite outgrowth of the chicken embryo DRG, we used follistatin, an activin-binding protein, to neutralize the bioactivities of activin A. As shown in Fig. 4A, activin A induced obvious neurite outgrowth, whereas no neurite outgrowth was observed in the follistatin-treated group (Fig. 4B). Furthermore, we found that follistatin greatly inhibited activin A-induced neurite outgrowth (Fig. 4C), but did not inhibit NGF-induced neurite outgrowth from DRG (Fig. 4D). Our data showed that the blockade of activin A action by anti-ActRIIA antibody also inhibited activin A-induced neurite outgrowth (supplementary material Fig. S1).

We subsequently acquired visible images of the neurite outgrowth under phase optics and analyzed the data on day 3. In order to measure the length of neurites clearly and accurately, we stained the cultured DRG with Gimsa (supplementary material Fig. S2). The data showed that in the positive control group (4 ng/ml NGF), the average length of neurites was 124.5±23.7 μm. Activin A also induced neurite extension from the DRG and the average length of neurites (5 ng/ml activin A) was 114.8±19.6 μm. Statistical analysis showed that there were significant increases of the neurite length and neurite count (Fig. 4E,F) in the activin A group and the NGF group ($P<0.01$),

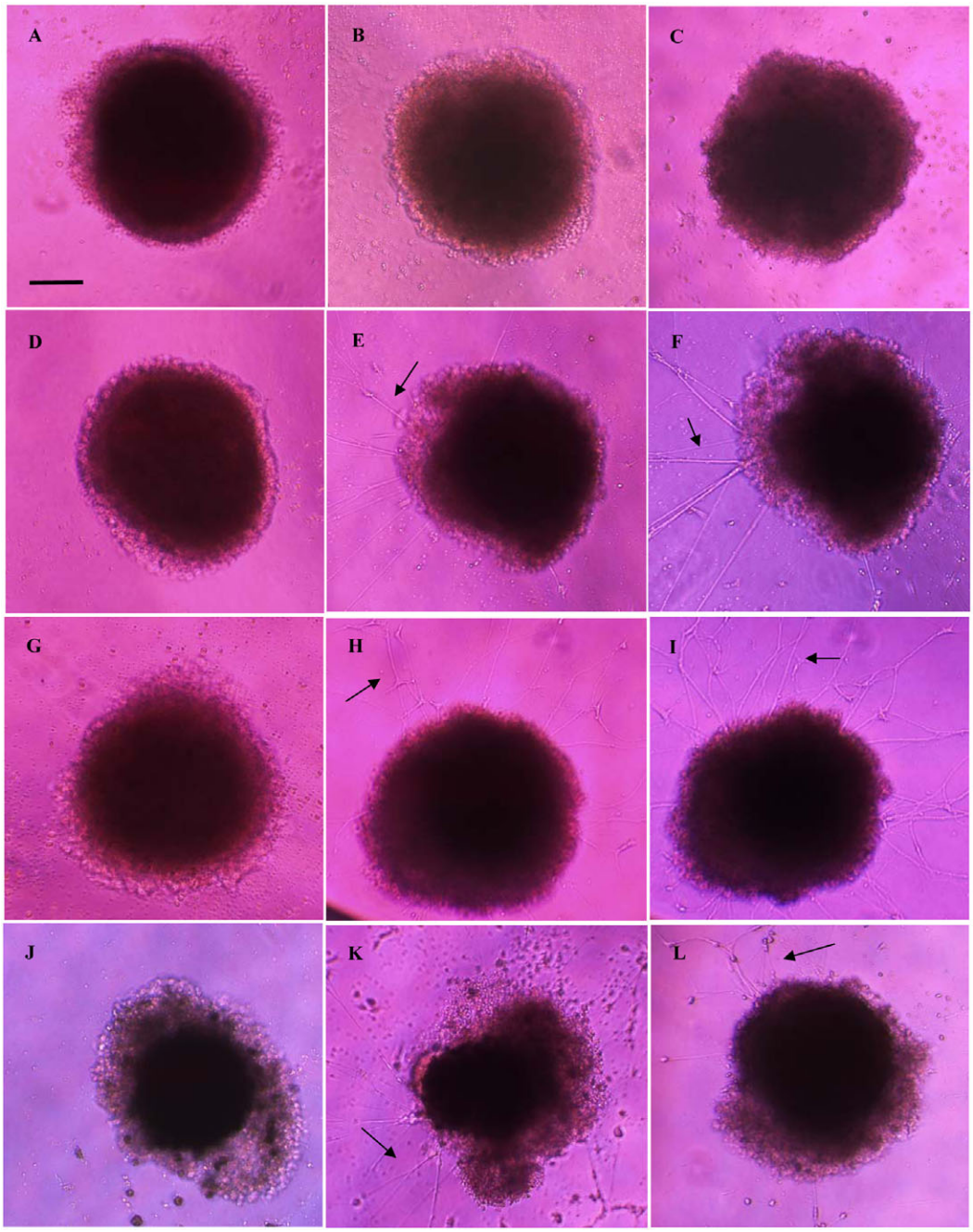


Fig. 3. DRG neurite outgrowth induced by activin A and NGF. (**A**–**C**) In the control culture with 0.5% FCS-DMEM medium, no neurites could be seen at 1 day (A), 2 days (B) and 3 days (C). (**D**–**F**) In the presence of 5 ng/ml activin A, significant neurite outgrowth was observed at 1 day (D), 2 days (E) and 3 days (F). (**G**–**I**) In the presence of 4 ng/ml NGF, marked neurite outgrowth was observed at 1 day (G) , 2 days (H) and 3 days (I). (**J**–**L**) DRG were cultured in serum-free medium for 3 days and no neurites grew (J). In the presence of 4 ng/ml NGF (K) or 5 ng/ml activin A (L) outgrowth of a few thin neurites was observed. Representative images of three independent experiments are shown. Arrows indicate neurites. Scale bar: 50 μm.

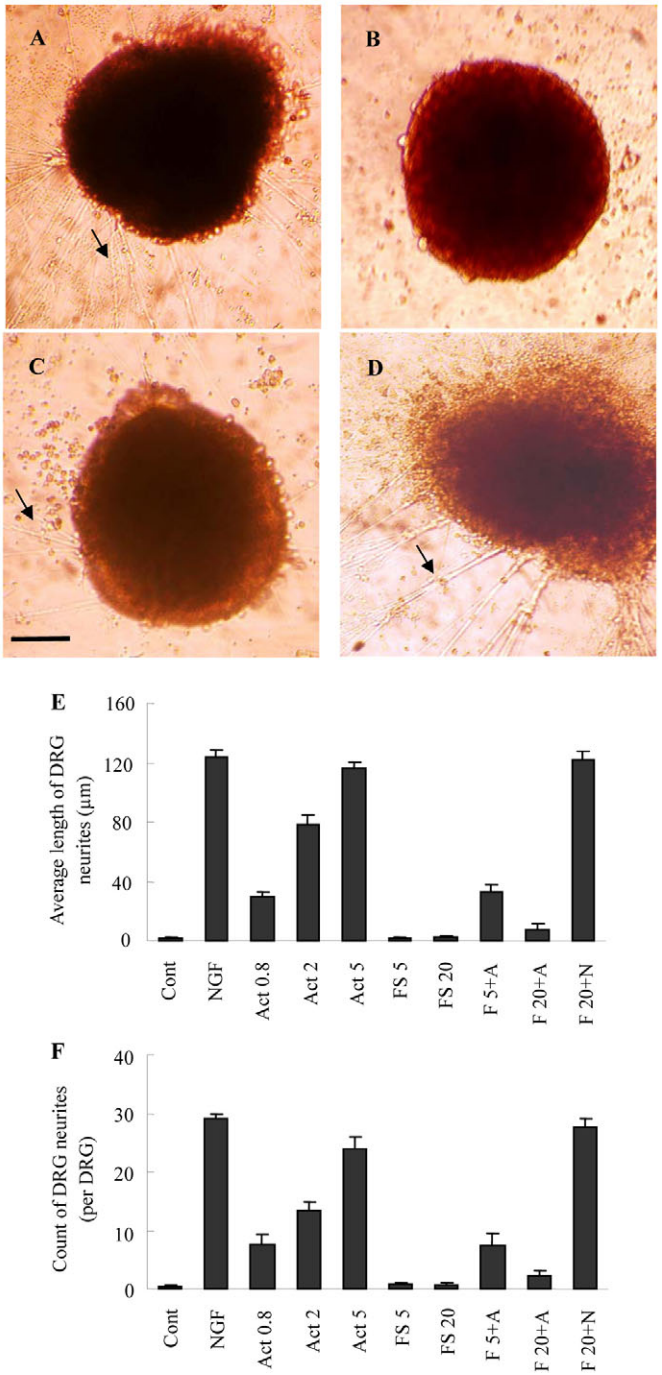
compared with the control group. Follistatin significantly inhibited activin A-induced neurite outgrowth, but did not alter neurite outgrowth induced by NGF.

Activin A maintains long-term survival of DRG neurons

The data described above clearly demonstrates the requirement for activin A during neurite outgrowth of DRG. To determine whether activin A has any effect on maintaining DRG neurons survival, monolayer-cultured ganglion cells of DRG were used to measure neuron survival. In control cultures, no living DRG neurons survived on day 10 (Fig. 5A), but we observed a large number of living DRG neurons in cultures treated with 4 ng/ml NGF (Fig. 5B). Surprisingly, in the 5 ng/ml activin A group we also found that many DRG neurons still survived on day 10 (Fig. 5C). Furthermore, although very few DRG neurons survived in the 20 ng/ml follistatin + 5 ng/ml activin A group

(Fig. 5E), a large number of DRG neurons were observed in the 20 ng/ml follistatin + 4 ng/ml NGF group on day 10 (Fig. 5F). Nissl body staining with Toluidine Blue was also used to confirm the existence of neurons (supplementary material Fig. S3).

Microtubule-associated protein 2 (MAP2) is a marker of neurons, and glial fibrillary acidic protein (GFAP) is a marker of Schwann cells and astrocytes. To confirm that activin A maintains long-term survival of DRG neurons, the cell types present among the living ganglion cells were determined using dual-label immunohistochemistry staining for MAP2 and GFAP. MAP2-immunoreactive cells (Fig. 5G, red arrows), which represent neurons, and GFAP-immunoreactive cells (Fig. 5G, green arrows) that are morphologically more similar to Schwann cells than to astroctyes were present in the activin A group (Fig. 5G). Statistical analysis of cell viability indicated a significant difference between the control culture and the activin A group (Fig. 5H). Our data also showed that follistatin significantly inhibited the effect of activin A in maintaining DRG neuron survival, but follistatin could not influence the effect of NGF on DRG neuron survival.



Activin A affects mRNA expression of ActRII and neuropeptides

CGRP and VIP are important neuropeptides that are widely distributed in the central and peripheral nervous system. To determine how activin A affects neurite outgrowth of DRG, mRNA expression of ActRIIA, ActRIIB and the neuropeptides CGRP and VIP were analyzed by real-time quantitative RT-PCR. Analysis of mRNA levels showed that ActRIIA, ActRIIB, CGRP and VIP were detectable in DRGs (Fig. 6). In activin A-treated DRGs, mRNA expression of ActRIIA and CGRP increased greatly, but the mRNA expression of ActRIIB and VIP was almost unchanged. As expected, NGF markedly upregulated CGRP and VIP mRNA expression.

Activin A stimulates 5-HT secretion by DRG

As a neurotransmitter and neuromodulator, serotonin (5-HT) influences neurite outgrowth and synaptogenesis in the nervous systems of several species. In this study, 5-HT levels in the supernatant of cultured DRGs were analyzed by LC/MS. The results showed that 5-HT secretion levels increased significantly in the NGF-treated group, and also activin A promoted 5-HT secretion greatly (Fig. 7). These data suggest that both activin A and NGF might stimulate neurite outgrowth in DRG by inducing 5-HT secretion in a similar manner.

Discussion

Homo- and hetero-dimerization of two inhibin β subunits, βA and βB, form three forms of activins: activin A (βAβA), activin B (βBβB) and activin AB (βAβB) (DiMuccio et al., 2005; Liu, et al., 2006). Activin mRNA and proteins are widely distributed in embryonic and adult tissues (Ebert et al., 2007; Mousa et al., 2003; Roberts et al., 1996). Activin receptors belong to the family of serine/threonine kinase receptors, and two receptor types have been identified: type I and type II (Attisano et al., 2001; Shoji et al., 2000; Tsuchida et al., 2001). The type II receptors of

Fig. 4. Effects of follistatin on activin A-induced neurite outgrowth of DRG. (**A**) Neurite extension was found in DRG cultured in 5 ng/ml activin A for 3 days. (**B**) No neural process growth was observed in DRG treated with 20 ng/ml follistatin. (**C**) Very few neurites grew in DRG cultured in the presence of 20 ng/ml follistatin + 5 ng/ml activin A. (**D**) Significant neurite outgrowth was observed in DRG treated with 20 ng/ml follistatin + 4 ng/ml NGF. Scale bar: 50 μm. Arrows indicate neurites. (**E**,**F**) The neurite count (F) and neurite length (E) of cultured DRG of chicken embryos were statistically analyzed. Cont: DRG cultured in 0.5% FCS-DMEM for 3 days as control culture; NGF: DRG maintained with NGF (4 ng/ml) as positive control; Act 0.8, 2, 5: DRG incubated with activin A (0.8, 2, 5 ng/ml, respectively); FS 5, 20: DRG incubated with follistatin (5, 20ng/ml, respectively); F5, 20+A: DRG treated with follistatin (5 ng/ml) + activin A (5 ng/ml) or follistatin (20 ng/ml) + activin A (5 ng/ml), respectively. F 20+N: DRG maintained in follistatin (20 ng/ml) + NGF (4 ng/ml). Data are presented as mean ± s.d. of three independent experiments. $n=18$ ganglia.

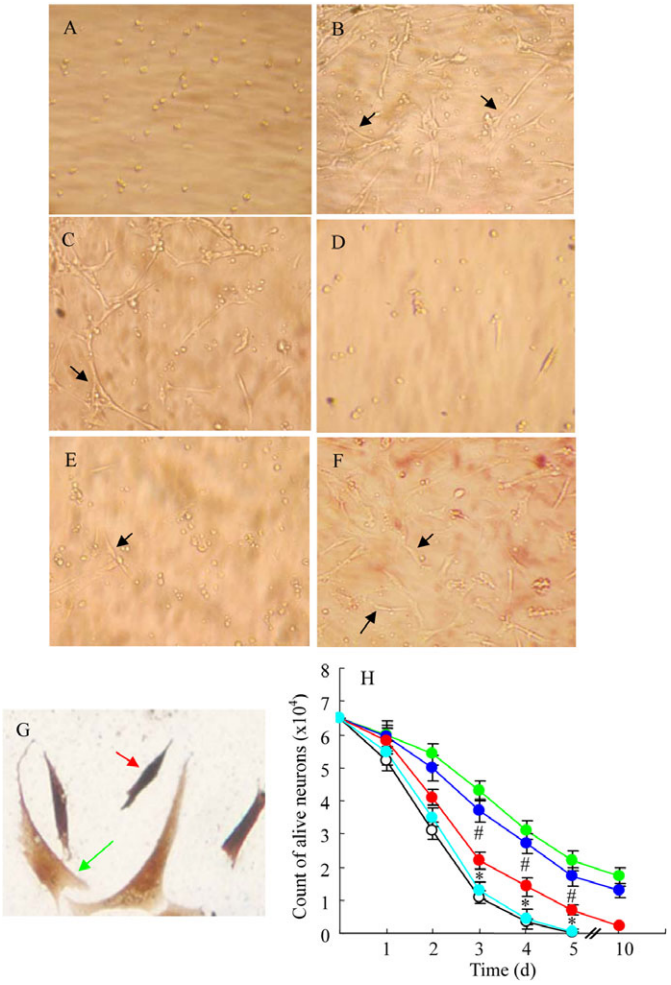


Fig. 5. Survival of embryonic DRG neurons cultured with activin A and NGF. (**A**) No living DRG neurons were found in the control culture (monolayer-cultured DRG neurons with 0.5% FCS-DMEM) on day 10. (**B**) Some viable neurons were observed in the positive control (4 ng/ml NGF) on day 10. (**C**) Some neurons could be found in the 5 ng/ml activin A-treated group on day 10. (**D**) No living neurons were observed in the 20 ng/ml follistatin-treated group on day 10. (**E**) There were very few neurons in the follistatin (20 ng/ml) + activin A (5 ng/ml) group on day 10. (**F**) Some neurons could be observed in the follistatin (20 ng/ml) + NGF (4 ng/ml) group on day 10. Arrows indicate living DRG neurons. Magnification: 100×. (**G**) The ganglion cell type was determined by dual-label immunohistochemical staining for MAP2 (dark staining) and GFAP (yellow staining). Green arrows indicate non-neurons and red arrows indicate neurons. Magnification: 1000×. (**H**) Time course of cell viability assay of cultured DRG neurons. The neurons were cultured in 0.5% FCS-DMEM as control culture (white circles), 4 ng/ml NGF (green circles), 5 ng/ml activin A (dark blue circles), 20 ng/ml follistatin (turquoise circles) or 20 ng/ml follistatin + 5 ng/ml activin A (red circles). Data are presented as mean ± s.d. $^{\#}P<0.01$, compared with culture control group; $^{*}P<0.01$, compared with 5 ng/ml activin A group.

activin have two subtypes (ActRIIA and ActRIIB), which are encoded by individual genes.

Activin A has a broad range of physiological activities, including maintaining neuron survival and protecting neurons from neurotoxic injury (Ge et al., 2010; Iwahori et al., 1997). Previous studies have reported that the neurotrophic effect of

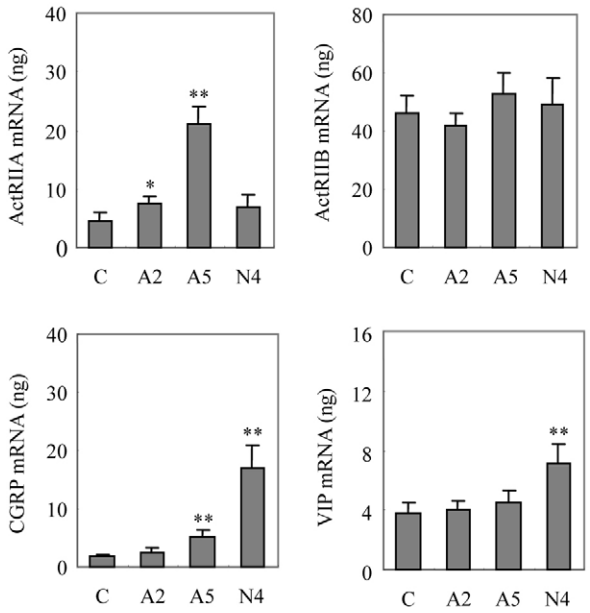


Fig. 6. ActRII and neuropeptides mRNA expression. mRNA expression of ActRIIA, ActRIIB, CGRP and VIP in DRG were analyzed by real-time quantitative RT-PCR. DRGs were incubated for 24 hours with 0.5% FCS-DMEM medium in the presence of activin A or NGF. The graph shows ActRII and neuropeptides mRNA levels. C, culture medium control; A2, 2 ng/ml Activin A; A5, 5 ng/ml Activin A; N4, 4 ng/ml NGF. $^{*}P<0.05$, $^{**}P<0.01$, compared with culture medium control. Data are presented as mean ± s.d.

activin A on midbrain dopaminergic neurons raises hopes for a rational therapeutic approach to Huntington's disease (Hughes et al., 1999b), and activin A is essential for neurogenesis following neurodegeneration (Abdipranoto-Cowley et al., 2009) and increases the number of synaptic contacts and the length of dendritic spine necks by modulating spinal actin dynamics (Shoji-Kasai et al., 2007). In order to evaluate the effect of activin A on neurite outgrowth of DRG neurons, primary cultured DRG of chicken embryos was used to test activin A-induced neurite outgrowth, a standard method to evaluate the biological activity of nerve growth and differentiation factors (Goncharova et al., 1987). We found that, not only ActRIIA and ActRIIB, but also activin βA mRNA expression were present in chicken DRG

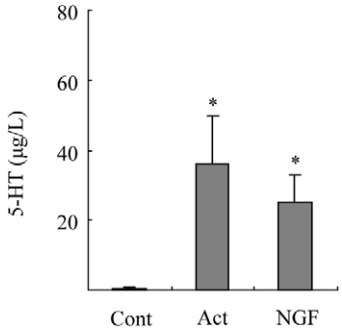


Fig. 7. 5-HT levels in the supernatant of cultured DRG. DRGs were incubated for 48 hours in 0.5% FCS-DMEM medium (Cont), 5 ng/ml activin A (Act) or 4ng/ml NGF. $^{*}P<0.01$, compared with culture control. Data are presented as mean ± s.d. $^{*}P<0.001$.

collected at embryonic day 8 (E8). Activin A significantly induced neurite outgrowth from DRG of chicken embryos. Neurites were long and flourishing in the DRGs treated with 5 ng/ml activin A, whereas no neurite outgrowth was found in the control group (Fig. 3). These data indicate that activin A might play an important role in neurite outgrowth of DRG in an autocrine or paracrine manner.

Previous reports have revealed that activin A participates in the regulation of neuron survival in vitro and in vivo (Funaba et al., 1997; Maira et al., 2010; Suzuki et al., 2010; Wankell et al., 2003). For example, activin A was shown to be a potent survival factor by promoting the survival of the B50 nerve cell line and EIO chick neural retina cell (Schubert et al., 1990), and exposure of primary cultures of rat hippocampal neurons to activin supported neuron survival (Iwahori et al., 1997). To investigate further the neurotrophic roles of activin A in the peripheral nervous system, the viability of chicken embryo DRG neurons sustained by activin A was tested using primary cultured DRG neurons. We found that activin A could maintain the long-term survival of DRG neurons during the observation period of 10 days, suggesting that activin A also has a neurotrophic effect on DRG neuron survival.

To confirm the hypothesis that activin A might stimulate neurite outgrowth of embryonic DRG of the chicken, we used follistatin (FS), an activin-binding protein, to neutralize the bioactivities of activin. FS can specifically bind activin as a soluble protein (Nakamura et al., 1990), appearing to act by blocking the binding of activin to ActRII and directly neutralizing the biological activity of activin, and can regulate multiple physiological functions in vivo (Winter et al., 1996; Sidis et al., 2001). Expression of the mRNA encoding FS has been detected in the brain of adult rats (Phillips et al., 1998). Previous studies focused on the detection of activin A or FS expression in diverse tissues to determine in which organs FS–activin interactions might be important for cell proliferation and differentiation. The regulatory effects of FS and activin on inflammation, acute phase response, tissue repair and liver cirrhosis (Phillips et al., 2009; Wang et al., 2008; Werner et al., 2006; Zhou et al., 2009), and their roles in central nervous system (MacConell et al., 1996; Phillips et al., 1998) have been reported. In this study, our data indicates that FS not only inhibited the DRG neurite outgrowth induced by activin A, but also restrained the DRG neuron survival maintained by activin A, whereas it did not alter NGF-induced neurite outgrowth from DRG neurons. Furthermore, we used an anti-ActRIIA antibody to block the binding of activin A with its receptor, and found that application of this antibody significantly inhibited the extension of neurites from activin A-induced DRG, but did not alter the neurite outgrowth of DRG stimulated by NGF. Taken together, we conclude from these studies that activin A can induce neurite outgrowth from DRG of chicken embryo.

In order to investigate the possible mechanism of activin A functions, mRNA expression of ActRII and neuropeptide in DRGs were examined using RT-PCR. ActRII can be expressed during the embryonic development of chicken DRG (Kos et al., 2001). Our data showed that expression of ActRIIA mRNA increased significantly in activin A-treated DRGs, but ActRIIB mRNA expression did not change. CGRP and its receptors are widely distributed in the central and peripheral nervous system (Ghatta and Nimmagadda, 2004; Ma et al., 2003). Previous studies had demonstrated that CGRP has a neuroprotective action and that upregulation of alpha-CGRP correlates with a possibility of axon regeneration (Li et al., 2004; Piehl et al., 1998; Wimalawansa, 1996). VIP is also a neuropeptide, and activin treatment of rat sympathetic neurons or cells from a neural crest-derived human neuroblastoma cell line results in upregulation of VIP expression (Symes et al., 2000). In the present study, we found that expression of CGRP mRNA in the activin A-induced DRG increased significantly, whereas VIP mRNA expression was not altered. As a neurotransmitter and neuromodulator, 5-HT influences neuronal outgrowth and synaptogenesis in vertebrates and invertebrates (Lima et al., 1994; Mercer et al., 1996; Schachtner et al., 1999; Zhou and Cohan, 2001). Our present study showed that 5-HT levels increased significantly in activin A-treated DRGs. These data indicate that 5-HT and CGRP might mediate the effect of activin A on neurite outgrowth and neuron survival of embryonic DRG.

In conclusion, activin A can stimulate neurite outgrowth of DRG neurons of the chicken by upregulating CGRP expression and 5-HT release, suggesting the possibility that activin A might be involved in induction of neurite outgrowth during nervous system development and could be a potential new drug target for diseases of the nervous system.

Materials and Methods

Animals

White Leghorn chicken E8 embryos were purchased from Breeding Bird Farms, Changchun, China. All procedures were approved by the Experimental Animal Center of Jilin University in accordance with the policies established in the Chinese Guide to Care and Use of Experimental Animals. Both the number of animals used and their suffering were minimized.

Immunohistochemical staining for activin A

DRGs were collected and fixed with 4% paraformaldehyde for 24 hours at room temperature (RT), embedded in paraffin, and then 3 μm-thick sections were mounted on slides. After dewaxing and rehydration, sections were incubated with 3% hydrogen peroxide (H_2O_2) in methanol to block endogenous peroxidase for 30 minutes at RT. Nonspecific reactivity was blocked by a preincubation of sections in 3% bovine serum albumin (BSA) for 30 minutes in 0.01 mol/l PBS containing 0.1% Triton X-100. Sections were incubated in rabbit anti-activin A polyclonal antibody (R&D, USA) overnight at 4℃, washed three times with PBS and then processed with biotinylated secondary antibodies for 10 minutes at RT. Sections were washed three times with PBS and incubated in streptomycete-horseradish peroxidase for 10 minutes at RT. After the sections were washed three times with PBS, immunoreactive products were visualized in 0.03% H_2O_2 and 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB), and then counterstained with Hematoxylin. Sections were dehydrated, cleared, counted and observed under an Olympus microscope (BX51). Images were taken with a DP72 camera (Olympus, Tokyo, Japan). For control staining, sections were incubated with normal rabbit IgG instead of anti-activin A antibody.

Culture of DRG

DRG were isolated from the lumbar regions of 48 embryos and collected by a mechanical treatment under a dissecting microscope as previously described (Kos et al., 2001). DRGs were plated onto poly-L-lysine (Sigma, USA) coated 48-well tissue culture plates. Each well contained six collected DRGs, and the DRGs were incubated for 1–3 minutes to allow them to attach to the substrate plate at 37℃ without culture medium. Then 200 μl Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Rockville, MD, USA) was added to each well, which contained 0.5% heat-inactivated fetal calf serum (FCS) in the presence of activin A (R&D, Minneapolis, MN, USA) (0.8 ng/ml, 2 ng/ml, 5 ng/ml), mouse nerve growth factor (NGF) (Wuhan Hiteck Bio-pharmaceutical Co., Wuhan, China) (4 ng/ml), or FS (5 ng/ml, 20 ng/ml), respectively. Tissue culture plates were incubated at 37℃ with 5% CO_2 and water-saturated air.

Assessment of digital images of neurite outgrowth

To quantify neurite outgrowth of DRG, we observed neurite outgrowth under phase optics using an inverted microscope (Leica, Wetzlar, Germany) and digital images were acquired using a DC3.7V digital camera (Olympus corporation, Japan). Neurite outgrowth was statistically assayed on day 3. The numbers of neurites were counted and the mean length of the five longest neurites of each ganglion was measured. For each experimental group, the length of neurites was

measured by connecting the end point of the neurite and the neuron soma, which was taken as the origin point of neurites (Hou et al., 2006).

Primary culture of DRG neurons

Dissociated DRGs were dispersed with collagenase and trypsin as described (Bi et al., 2006). The cell clusters were gently triturated with fire-polished Pasteur pipettes, and then centrifuged at 600 ***g*** for 5 minutes. The supernatant was discarded and the cell pellet was rinsed three times with 0.01 mol/l phosphate-buffered saline (PBS), pH 7.4. The cells were resuspended and plated onto poly-L-lysine-coated 48-well culture plates (6.5×10^4 per well) with 0.5% FCS-DMEM in the presence of activin A (5 ng/ml) or NGF (4 ng/ml). The cells were cultured at 37℃ with 5% CO_2 and the culture media were exchanged every three days. Cell viabilities were observed under phase optics using an inverted microscope (Leica, Wetzlar, Germany) and digital images were acquired.

DRG neuron viability assay

To determine the number of living ganglion cells, the viability of ganglion cells of DRG was detected using the Trypan Blue exclusion method and the living ganglion cells were counted for each sample under high magnification using a blood counting chamber. As Nissl bodies are a marker of neurons (Schwartz, 1986), the proportion of cells with Nissl bodies (stained with Toluidine Blue) was analyzed in the 200 ganglion cells counted. The DRG neuron number was finally determined as follows.

Dual-label immunohistochemistry staining for MAP2 and GFAP

To distinguish between neurons and non-neurons, the cultured ganglion cells were fixed with 4% paraformaldehyde for 30 minutes, blocked with 2% BSA and incubated in 0.1% Triton-X-100 for 10 minutes, and then stained for MAP2 using a goat anti-MAP2 antibody (1:100; BD Biosciences, UK) at 4℃ overnight. After being washed with PBS three times, the slides were incubated with alkaline phosphatase (AP)-conjugated mouse anti-goat IgG antibody (1:400; Santa Cruz, CA, USA) at 37℃ for 1 hour. Slides were then washed three times and incubated in 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (BCIP/NBT) staining kit for the subsequent staining procedures according to the manufacturer's protocol (BSD Biotechnology, Wuhan, China). After washing, slides were stained for GFAP using a rabbit anti-GFAP antibody (1:100; Serotec, Oxford, UK) at 37℃ for 1 hour. Horseradish peroxidase (HRP)-conjugated mouse anti-rabbit IgG antibody (1:400; Sigma, St Louis, MO, USA) was added and the slides were incubated at 37℃ for 30 minutes. The slides were finally incubated in 0.05% DAB and 0.01% hydrogen peroxide for 5 minutes, and then washed with PBS three times. The cultures underwent gradient alcoholic dehydration followed by clearing with xylene, then mounted with gum and observed under the light microscope.

Real-time quantitative RT-PCR

To detect ActRIIA, ActRIIB, CGRP and VIP mRNA expression levels in DRG, total RNA from DRGs was extracted by using the TRIzol reagent according to the manufacturer's protocol (Invitrogen, UK). The fluorescence quantitative RT-PCR reagents were purchased from Takara Biotechnology Co (Kyoto, Japan), and real-time quantitative RT-PCR was performed on the ABI PRISM 7300 (Perkin-Elmer Applied Biosystems) in a two-stage, single-tube reaction as described previously (Wang et al. 2008). Primers used were as follows: ActRIIA sense primer, 5′-ACGATTTGGGTGTGTGTGGAAAG-3′; ActRIIA antisense primer, 5′-TGTGC-CAACCTGTCCATGTGTATC-3′; ActRIIB sense primer, 5′-TATATGAGCCG-CCACCACCAAC-3′; ActRIIB antisense primer, 5′-TTGCCCTTCAGGTAA-TCCGTG-3′; CGRP sense primer, 5′-CTGCAGCCTGGATAGACCTA-3′; CGRP antisense primer, 5′-CAGGCACAAAAAGAGTCTACG-3′; VIP sense primer, 5′-AGTCCTGTCAAACGCCACTC-3′; VIP antisense primer, 5′-TTCA-GAGGTCCAATGGGAGG-3′; GAPDH sense primer, 5′-GTCCAAGTGGTGG-CCATCAA-3′; GAPDH antisense primer, 5′-GCTGAGGGAGCTGAGATGAT-3′. The RT-PCR products were quantitatively analyzed according to the standard mRNA calibration curve.

Determination of 5-HT levels

Forty ganglia were plated onto each well of poly-L-lysine-coated 48-well tissue culture plates in 200 µl 0.5% FCS-DMEM culture medium, and then the supernatant of the cultured DRG was collected after two days. Neurotransmitter 5-HT secretion levels in the supernatant of cultured DRG were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS) (Miller et al., 2010; Uutela et al., 2009). Cold TCA (300 µl) was added to each sample and then mixed for 30 seconds using a vortex. Samples were centrifuged for 5 minutes (15,000 ***g***) and the supernatant was added into the chromatographic column of LC-MS/MS system. The chromatographic column was a ZoBax SB-C8 column 4.6×150 mm (5 µm). The mobile phase contained 5 mmol/l acetonitrile and ammonium acetate buffer (20:80, v/v). The flow rate was 0.8 ml/min and the column temperature was 30℃. MS conditions were as follows: ion spray voltages 3000 V, collision energy 25 eV. Nitrogen was used as a nebulizer (50 psi). Mass spectrometric detection in a positive ion mode was carried out using multiple reaction-monitoring (MRM). Ion transitions were (m/z 177 → m/z 160).

Statistical analysis

GraphPad Prism 4.0 Software (GraphPad, San Diego, California, USA) was used to perform statistical analyses and graphical presentation. Data were expressed as mean ± s.d., and analyzed by two-tailed one-way ANOVA followed by Bonferroni's multiple comparison test.

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