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

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Running title: Activin A induces DRG neurite outgrowth
Summary

Activin A, a member of transforming growth factor β (TGF-β) superfamily, plays essential role in neuron survival as a neurotrophic and neuroprotective factor in central nervous system. However, the effects and mechanisms 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 day 8 (E8). Moreover, activin A induced neurite outgrowth of the primary cultured DRG and maintained long-time survival of monolayer-cultured DRG neurons during the observation for 10 days. Follistatin (FS), an activin-binding protein, significantly inhibited activin A-induced neurite outgrowth of DRG, but fails to influence the effect of nerve growth factor (NGF) on DRG neurite outgrowth. Furthermore, the results showed that activin A significantly up-regulated mRNA expressions 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 may induce DRG neurite outgrowth via promoting CGRP expression and stimulating 5-HT release. These data suggest that activin A plays an important role in the development of DRG by autocrine/paracrine manner.

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

Activin A, a member of transforming growth factor β (TGF-β) 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 anterior pituitary (Liu et al., 1996), spermatogenesis (Mather et al., 1990), stimulation of erythroblasts 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 cultured neuron survival of central nervous system (CNS) 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 the expressions of various kinds of growth and differentiation factors were up-regulated after brain injury, including the increase of 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 the dorsal root ganglia (DRG) in peripheral nervous system.

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 damage-related regions of the hippocampus is likely to be an important, and possibly a protective component in 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 day 8 (E8). Meanwhile, type II receptor of activin (ActRII) and neuropeptides expressions and 5-HT release were analyzed 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 role have been reported in central nervous system and its receptors’ expression has been observed in peripheral nervous system [Kos et al., 2001]. We therefore sought to determine whether activin A is expressed in peripheral nervous system. By reverse transcription polymerase chain reaction (RT-PCR), not only type IIA and IIB receptor of activin (ActRIIA and ActRIIB), but also activin βA mRNA expressions were detected in chicken DRG (Fig. 1A). Furthermore, mature activin A protein expression was also detectable in DRG by immunohistochemical staining (Fig. 1B). These data suggested that activin A might play an important role in DRG by autocrine/paracrine manner.

**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 peripheral nervous system by culturing primary DRG and observing neurite outgrowth. We observed neurite outgrowth from cultured DRG in groups of 10\% FCS-DMEM and 2.5\% FCS-DMEM in vitro for 3 days (Fig. 2A, 2B). However, no neurite extension was found in group of 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. Since NGF can promote DRG neurogenesis and neurite growth (Levi et al., 1996, Kashiba et al., 1998; Ma et al., 1999; Rifkin et al., 2000), it was applied in our present study as a positive control. Although neurite outgrowth from cultured DRG was undetectable when cultured in 0.5\% FCS-DMEM, we observed obvious neurite outgrowth from the DRG when NGF was added in the culture (Fig. 2D). Therefore 0.5\% FCS-DMEM was chosen to examine neurite outgrowth of embryonic DRG in the following studies.

**Activin A stimulates neurite outgrowth of DRG**

We tested the effect of activin A by the culture system established above. Though no neurite extension could be observed in DRG when cultured in 0.5\% FCS-DMEM (Fig 3A, 3B, 3C), we noted obvious neurite outgrowth in DRG cultured with 5ng/ml activin A (Fig. 3D, 3E, 3F) or 4ng/ml NGF (Fig. 3G, 3H, 3I). To better confirm activin A-induced DRG neurite outgrowth, the DRG was further cultured in serum
free medium. We also found that no neurite grew in serum free medium cultured DRG for 3 days, but in 4 ng/ml NGF and 5 ng/ml activin A groups showed the thin neurite outgrowth of embryonic DRG of chicken (Fig. 3J, 3K, 3L). These results suggest that activin A had similar role to the 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 Figure 4A, activin A induced the obvious neurite outgrowth of DRG, while no neurite outgrowth was observed in the group of follistatin (Fig. 4B). Furthermore, we found that follistatin greatly inhibited activin A-induced neurite outgrowth (Fig. 4C), but follistatin did not inhibit NGF-induced neurite outgrowth from DRG (Fig. 4D).

We subsequently acquired visible images of the neurite outgrowth under phase optics and analyzed the data on day 3. 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 the 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 and 4F) in the activin A group and NGF group (p<0.01), compared with control group. Follistatin significantly inhibited activin A-induced neurite outgrowth, but did not alter neurite
outgrowth induced by NGF.

**Activin A maintains long-time survival of DRG neurons.**

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

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

**Activin A affects mRNA expressions of ActRII and neuropeptides**

CGRP and VIP are important neuropeptides which are widely distributed in the central and peripheral nervous system. To determine effect mechanism of activin A on neurite outgrowth of DRG, mRNA expressions of ActRIIA, ActRIIB, 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 not altered. As expected, NGF markedly induced CGRP and VIP mRNA expression.

**Activin A stimulates 5-HT secretion of 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 NGF group, and also activin A promoted 5-HT secretion greatly (Fig. 7). The data suggest that activin A may stimulate neurite outgrowth of DRG via inducing 5-HT secretion by a similar set of responses with NGF.
Discussion

Homo- and hetero-dimerizations 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 II (Attisano et al., 2001; Shoji et al., 2000; Tsuchida et al., 2001). The type II receptors of 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 activin A on midbrain dopaminergic neurons raise hopes for a rational therapeutic approach to Huntington's disease (Hughes et al., 1999b), and activin A is essential for neurogenesis following neurodegeneration (Abdipranoto et al., 2009) and increases the number of synaptic contacts and the length of dendritic spine necks by modulating spinal actin dynamics (Shoji et al., 2007). In order to evaluate the effect of activin A on neurite outgrowth of DRG neurons, primary cultured DRG of chicken embryo was used to test activin A-induced neurite outgrowth, a standard method to evaluate the biological activity of nerve growth and differentiation factor (Goncharova et al., 1987). We found that not only ActRIIA and ActRIIB, but also activin βA mRNA
expression were present in chicken DRG collected from 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 5ng/ml activin A, whereas no neurite outgrowth was found in the control group (Fig. 3). These data indicate that activin A may play an important role in neurite outgrowth of DRG by an autocrine / 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 promoted the survival of the B50 nerve cell line and EIO chick neural retina cell as a potent survival factor (Schubert et al., 1990), and exposure of primary cultures of rat hippocampal neurons to activin supported neuron survival (Iwahori et al., 1997). To further investigate neurotrophic roles of activin A in peripheral nervous system, the viability of neurons of chicken embryo DRG sustained by activin A were tested by using the primary cultured DRG neurons. We found that activin A could maintain the long-time survival of DRG neurons during the observation period for 10 days, suggesting that activin A also has neurotrophic effect on DRG neuron survival.

To confirm 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), which seems to act via blocking the binding of activin with ActRII and directly neutralizing activin biological activity, and regulate
multi-physiologic function in vivo (Winter et al., 1996; Sidis et al., 2001). Its mRNA expression in the brain of adult rats has been detected (Phillips et al., 1998). Previous studies focused on the detection of activin A or FS expression in diverse tissues to determine that 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 indicated that FS not only inhibited DRG neurite outgrowth induced by activin A, but also restrained DRG neurons survival maintained by activin A, whereas it did not alter NGF-induced neurite outgrowth from DRG neurons. Furthermore, we used anti-ActRIIA antibody to block the binding of activin A with its receptor, and found that the application of anti-ActRIIA antibody significantly inhibited the extension of neurite 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 expressions of ActRII and neuropeptide in DRGs were examined by 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 up-regulation of alpha-CGRP best 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-de-rived human neuroblastoma cell line results in an up-regulation 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 indicated that 5-HT and CGRP might mediate the effect of activin A on stimulating neurite outgrowth and maintaining neuron survival of the embryonic DRG.

In conclusion, activin A can stimulate neurite outgrowth of DRG neurons of the chicken via up-regulating CGRP expression and 5-HT release, suggesting the possibility that activin A may be useful to induce neurite outgrowth during nervous development and supporting a new clue in curing nervous system disease of activin A.

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 h at room temperature (RT), embedded in paraffin, and then 3-μm sections were mounted on slides. After dewaxing and rehydration, the sections were incubated with 3% hydrogen peroxide (H₂O₂)-methanol to block endogenous peroxidase for 30 min at RT. Nonspecific reactivity was blocked by a preincubation of sections in 3% bovine serum albumin (BSA) for 30 min 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 4ºC, washed three times with PBS and then processed with biotinylated secondary antibodies for 10 min at RT. The sections were washed three times with PBS and incubated in streptomyecete-horseradish peroxidase for 10 min at RT. After the sections were washed three times with PBS, immunoreactive products were visualized in 0.03% H₂O₂ and 0.05% diaminobenzidine (DAB), and then haematoxylin counterstain was used as backstaining. The sections were dehydrated, cleared, counted and observed under Olympus microscope (BX51). The photographs 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 min to allow them to attach to the substrate plate at 37 °C without culture medium. Then 200μl Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Rockville, MD, USA) was added in each well, which contained 0.5% heat-inactivated fetal calf serum (FCS) in the presence of activin A (R&D, Minneapolis, MN USA) (0.8ng/ml, 2ng/ml, 5ng/ml), mouse nerve growth factor (NGF) (Hiteck Biological Pharma Co Wuhan, China) (4ng/ml), or FS (5ng/ml, 20ng/ml), respectively. Tissue culture plates were incubated at 37°C with 5% CO₂ and water saturated air.

**Assay of digital images of neurite outgrowth**

To further verify the quantificational neurite outgrowth of DRG, we observed neurite outgrowth under phase optics by using an inverted microscope (Leica, Wetzlar, Germany) and digital images were acquired with digital camera DC3.7V (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 neurite was
measured by connecting the end point of the neurite and neuron soma, which was taken as the origin point of neurites (Hou et al., 2006).

**Primary culture of DRG neurons**

The 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 min. The supernatant was discarded and the cell pellet was rinsed with pH7.4, 0.01 mol/L phosphate-buffered saline (PBS) for 3 times. The cells were resuspended and plated on 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 (5ng/ml) and NGF (4ng/ml), respectively. The cells were cultured at 37°C with 5% CO₂ and the culture media were exchanged every three days. Cell viabilities were observed under phase optics by using an inverted microscope (Leica, Wetzlar, Germany) and digital images were acquired.

**DRG neurons viability assay**

To assay the number of living ganglion cells, the viability of ganglion cells of DRG was detected by trypan blue exclusion method and the living ganglion cells were counted for each sample under the high magnification using blood counting chamber. Since Nissl bodies are the marker of neurons (Schwartz, 1986), ratio of cells of Nissl bodies stained with toluidine blue was analyzed in the counted 200 ganglion cells. The DRG neuron number was finally determined as follow.
Dual-label immunohistochemistry staining for MAP2 and GFAP

To distinguish neurons and non-neurons, the cultured ganglion cells were fixed with 4% paraformaldehyde for 30 min, blocked with 2% bovine serum albumin (BSA) and incubated in 0.1% Triton-X-100 for 10 min, and then stained for MAP2 with a goat anti-MAP2 antibody (1:100; BD Biosciences, UK) at 4 °C overnight. After being washed with PBS for three times, the slides were incubated with Alkaline phosphatase (AP)-conjugated-mouse anti-goat IgG antibody (1:400; Inc, Santa Cruz, CA, USA) at 37 °C for 1 h. Then the slides were 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. Inc, Wuhan, China). After washing, slides were stained for GFAP by using a rabbit anti-GFAP antibody (1:100; Serotec, Oxford, UK) at 37 °C for 1 h. 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 °C for 30 min. The slides were finally incubated in 0.05% 3,3’-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide for 5 min, and then slides were washed with PBS for three times. The cultures underwent gradient alcoholic dehydration followed by clearing with xylene, and mounting with gum were carried out 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). The set of primers was designed as follows: ActRIIA sense primer 5’-acgatttgggtgttgagaag -3’, and the antisense primer 5’- tgtgccaacctgtctgttcat -3’; ActRIIB sense primer 5’- tatatgagccgcactgtcact -3’, and the antisense primer 5’- ttgccctcaggtaatcggggtgttgagaag -3’; CGRP sense primer 5’- ctgcagcctggatagaccta -3’, and the antisense primer 5’- caggcacaaaaagagttacgctg -3’; VIP sense primer 5’- agtcctgtcaaacgccactc -3’, and the antisense primer 5’- ttcagaggtccaatggagggttgagaag -3’; GAPDH sense primer 5’- gtccaagtggtggccatcaa -3’ and the 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 0.5% FCS-DMEM culture medium 200μl, and then the supernatant of 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
(300μL) was added to each sample and then vortexmixed for 30 seconds. 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). 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°C. The MS conditions were as follows: the ion spray voltages 3000v, collision energy 25eV. 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).

**Statistic analysis**

GraphPad Prism 4.0 Software (GraphPad, San Diego, California, USA) was used to perform statistic analyses and graphical presentation. Data were expressed with means ± S.D, and analyzed by two-tailed one-way ANOVA followed by Bonferroni’s multiple comparison test.

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FIGURE LEGENDS

Fig. 1. Activin βA and its receptor expression in chicken DRG. (A) Activin βA, ActRIIA and ActRIIB mRNA expressions were examined by RT-PCR. Lane1, molecular weight marker; Lane2, activin βA; Lane3, ActRIIB; Lane4, ActRIIA; Lane5, 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.

Fig. 2. Neurite outgrowth of the cultured DRG of chicken embryos in FCS-dependent manner. (A) A significant neurite outgrowth from DRG cultured with 10%FCS-DMEM was observed at 37°C with 5% CO₂ for 3 days. (B) Some neurite from DRG incubated with 2.5%FCS-DMEM grew. (C) The neurite outgrowth was not found in DRG maintained with 0.5% FCS-DMEM. (D) The significant neurite outgrowth was observed in the positive control DRG incubated in 0.5% FCS-DMEM containing 4ng/ml NGF for 3 days. Scale bar=50 μm. Arrows represent neuritis.

Fig. 3. Neurite outgrowth of DRG induced by activin A and NGF. (A)-(C) In the culture control with 0.5% FCS-DMEM medium, no neurites could be observed for 1 day, 2 days and 3 days, respectively. (D)-(F) In the presence of 5 ng/ml activin A, the significant neurite outgrowth was found for 1 day, 2 days and 3 days, respectively. (G)-(I) In the presence of 4 ng/ml NGF, the marked neurite outgrowth was observed for 1 day, 2 days and 3 days, respectively. (J)-(L) DRG was cultured in serum free
medium for day 3 and no neurite grew (J), in 4 ng/ml NGF (K) and 5 ng/ml activin A (L) showed few and thin neurite outgrowth. A representative value of the three independent experiments is shown. Arrows represent neurites. Scale bar=50 μm.

**Fig. 4.** Effects of Follistatin on blocking activin A-induced neurite outgrowth of DRG. (A) The 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 20ng/ml follistatin. (C) Very fewer neurite outgrowth grew in DRG cultured in the presence of 20ng/ml follistatin + 5ng/ml activin A. (D) The significant neurite outgrowth was observed in DRG treated with 20 ng/ml follistatin + 4 ng/ml NGF. Scale bar=50μm. The neurite count (E) and neurite length (F) of the cultured DRG of chicken embryos were statistically analyzed. Cont: the DRG was cultured in 0.5% FCS-DMEM for 3 days as culture control; NGF: the DRG was maintained with NGF (4ng/ml) as positive control; Act 0.8, 2, 5: the DRG was incubated with activin A (0.8, 2, 5ng/ml), respectively; FS 5, 20: the DRG was incubated with follistatin (5, 20ng/ml), respectively; F5, 20+A: the DRG was treated with follistatin (5ng/ml) + activin A (5ng/ml) and follistatin (20ng/ml) + activin A (5ng/ml), respectively. F 20+N: the DRG was maintained in follistatin (20ng/ml) + NGF (4ng/ml). Data are presented as mean ± SD of three independent experiments. n=18 (18 ganglia).

**Fig. 5.** Survival of embryonic DRG neurons cultured with activin A and NGF. (A) No living DRG neurons were found in culture control with 0.5% FCS-DMEM by
monolayer-cultured DRG neurons on day 10. (B) There was a part of neurons in the positive control with 4ng/ml NGF on day 10. (C) Some neurons could be found in the 5ng/ml activin A group on day 10. (D) No living neurons were observed in the 20ng/ml follistatin on day 10. (E) There were very fewer neurons in the follistatin (20ng/ml)+activin A (5ng/ml) group on day 10. (F) A part of of neurons could be observed in the follistatin (20ng/ml) + NGF (4ng/ml) group on day 10. Arrows represent living DRG neurons. Magnification: 100x. (G) The ganglion cells type was determined by Dual-label immunohistochemical staining for MAP2 (dark) and GFAP (yellow), respectively. Green arrows represent non-neurons and red arrows represent neurons. Magnification: 1000x. (H) Time course of cell viability assay of the cultured DRG neurons,. The neurons were cultured in 0.5% FCS-DMEM as culture control (○), 4ng/ml NGF (●), 5ng/ml activin A (●), 20ng/ml follistatin (●) and 20ng/ml follistatin + 5ng/ml activin A (●). *p<0.01, compared with culture control group; **p<0.01, compared with 5ng/ml activin A group.

**Fig. 6.** Assay of ActRII and neuropeptides mRNA expressions. The mRNA expressions of ActRIIA, ActRIIB, CGRP and VIP in DRG were analyzed by real-time quantitative RT-PCR. The DRGs were incubated for 24h with 0.5% FCS-DMEM medium in the presence of activin A or NGF. The graph showed the 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.
**Fig. 7.** The 5-HT levels in the supernatant of cultured DRG. The DRG's were incubated for 48h in 0.5% FCS-DMEM medium (Cont), 5ng/ml activin A (Act) and 4ng/ml NGF, respectively. *p<0.01, compared with culture control.
Fig. 1.

A

1 2 3 4 5

ActRIIB → ← ActRIIA
Activin βA → ← GAPDH

B

IgG Control Anti-Activin A
Fig. 2.
Fig. 4.

E

Average length of DRG neurites (µm)

Cont  NGF  Act 0.8  Act 2  Act 5  FS 5  FS 20  F 5-A  F 20-A  F 20-N

0  40  80  120  160

F

Count of DRG neurites (per DRG)

Cont  NGF  Act 0.8  Act 2  Act 5  FS 5  FS 20  F 5-A  F 20-A  F 20-N

0  10  20  30  40
Fig. 5.

(A) Count of alive neurons (x10^4) vs Time (d)

(B) Journal of Cell Science Accepted manuscript

(C) Journal of Cell Science Accepted manuscript

(D) Journal of Cell Science Accepted manuscript

(E) Journal of Cell Science Accepted manuscript

(F) Journal of Cell Science Accepted manuscript

(G) Journal of Cell Science Accepted manuscript

(H) Journal of Cell Science Accepted manuscript
Fig. 6.
Fig. 7.