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Original Article

β-adrenergic signaling promotes posteriorization in *Xenopus* early development

Shoko Mori,[1] Yuki Moriyama,[1] Kumiko Yoshikawa,[2] Tomoyo Furukawa[1] and Hiroki Kuroda[1,2]*

[1]*Graduate School of Science and Technology and* [2]*Department of Education (Sciences), Shizuoka University, 836 Ohya Suruga-ku, Shizuoka, 422-8529, Japan*

Adrenaline (also known as Epinephrine) is a hormone, which works as major regulator of various biological events such stages of vertebrate, the role of adrenaline for early embryogenesis has been as heart rate, blood vessel and air passage diameters, and metabolic shifts. Although its specific receptors are expressing at the early developmental stage those functions are poorly understood. Here, we show that loss-of-functional effects of adrenergic receptor β-2 (Adrβ2), which was known as the major receptor for adrenaline and highly expressed in embryonic stages, led posterior defects at the tadpole stage of *Xenopus* embryos, while embryos injected with *Adrβ2* mRNA or treated with adrenaline hormone adversely lost anterior structures. This posteriorization effect by adrenaline hormone was dose-dependently increased but effectively rescued by microinjection of antisense morpholino oligomer for Adrβ2 (Adrβ2-MO). Combination of adrenaline treatments and microinjection of *Adrβ2* mRNA maximized efficiency in its posteriorizing activity. Interestingly, both gain- and loss-of-functional treatment for β-adrenergic signaling could not influence anterior neural fate induced by overexpression of *Chordin* mRNA in presumptive ectodermal region, meaning that it worked via mesoderm. Taken together with these results, we conclude that adrenaline is a novel regulator of anteroposterior axis formation in vertebrates.

Key words: adrenaline, anteroposterior patterning, fibroblast growth factor, posteriorization, Wnt.

Introduction

During blastula and gastrula stages the establishment of anteroposterior (A–P) patterning of the amphibian embryo starts in response to signaling by several groups of secreted molecules. *Xenopus* anterior neural induction first occurs at blastula stage by bone morphogenetic protein (BMP) antagonists Chordin and Noggin, which are expressed at the dorsal-animal region, and this low-BMP region called the blastula Chordin- and Noggin-expressing (BCNE) center gives rise to anterior neural tissue and is indispensable for brain formation (Kuroda *et al.* 2004; Ishibashi *et al.* 2008). At gastrula stage cerberus, which is expressed in the anterior endoderm region and functions as multiantagonists to block transforming growth factor (TGF)-β molecules such as BMPs and nodals and canonical Wnt proteins (Niehrs 2010), is required for head formation (Bouwmeester *et al.* 1996). Retinoic acid and fibroblast growth factor (FGF) also functions as an important regulatory signaling for A–P patterning, and blockade of FGF signaling downregulates the expression of members of the RAR (retinoic acid receptor) signaling pathway, resulting in anteriorization of *Xenopus* embryos (Tannahill *et al.* 1992; Blumberg *et al.* 1997; Shiotsugu *et al.* 2004). Interestingly, Shisa, which is strongly expressed in the prospective head ectoderm and the Spemann organizer of *Xenopus* gastrula embryos, and physically interacts with immature forms of the Wnt receptor Frizzled and the FGF receptor within the endoplasmic reticulum. As a result Shisa inhibits their post-translational maturation and trafficking to the cell surface of Wnt and FGF, and Shisa therefore promotes head formation like cerberus (Yamamoto *et al.* 2005).

As a hormone and neurotransmitter, adrenaline acts on nearly all body tissues, and its actions are strongly dependent on tissue type and tissue expression of adrenergic receptors. Adrenaline acts by binding to a variety of adrenergic receptors and is a nonselective agonist of all adrenergic receptors, including the major

*Author to whom correspondence should be addressed.
Email: ehkurod@ipc.shizuoka.ac.jp
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subtypes α1, α2, β1, β2, and β3 (Cotecchia *et al.* 2012). β-adrenergic receptor (Adrβ) and their associated guanine nucleotide regulatory protein (G protein)/adenylyl cyclase signal transduction pathways are central to the overall regulation of cardiant function (Moniotte *et al.* 2001; Wachter & Gilbert 2012). Interestingly, in the early developmental stage of *Xenopus* embryos, RNA coding β1 receptor is present in the mature oocyte, decreases after fertilization up to stage six and then gradually increases during gastrulation (Devic *et al.* 1997), meaning that embryonic development is likely to be regulated by adrenaline. However, the role of adrenaline for early developmental stages has never been examined. This study is the first report of the role of β-adrenergic signaling for early developmental stage of vertebrate. We show that adrenaline and its receptor have posteriorizing activity during the gastrula stage.

Materials and methods

Embryo manipulations

Xenopus laevis eggs were obtained from females injected with 400 units of human chorionic gonadotropin (HCG, Fuji Pharma, Japan), fertilized *in vitro* with minced testis, and then cultured in 0.1× Steinberg's solution (SS). Frog embryos were always cultured at 22 °C. The jelly coats were removed with thioglycolic acid solution (1% of thioglycolic acid in 0.1× SS, pH 8.0). Embryos were fixed with PBSFA for 2 h, dehydrated with ethanol, embedded in paraffin wax, sectioned at 8 μm, and stained with hematoxylin and eosin (HE). The following primers were used for reverse transcription–polymerase chain reaction (RT–PCR); Adrβ1-RT-fw, 5′-CACCCTTCCGCTACCAGAGT-3′, Adrβ1-RT-rv, 5′-AGGACGTGGCTATGGGAGAA-3′, Adrβ2-RT-fw, 5′-GTGGTCATGATCTTCGTCTA-3′, Adrβ2-RT-rv, 5′-TTGGAACCTGGTTTGCGTAAG-3′, PNMT-RT-fw, 5′-GATGTTCATCAGCCCAATC-3′, PNMT-RT-rv, 5′-AAAACACCCCATTGACATC-3′, Six3-RT-fw, 5′-GCAACTTCAGGGAGCTCTAC-3′, Six3-RT-rv, 5′-TAGGGATCCTGCAAGTACCA-3′, Rx2a-RT-fw: 5′-TAGTCTTCCTCTGGACTCCT-3′, Rx2a-RT-rv, 5′-CCGAAAGACTGGATGTGTTC-3′, Engrailed-RT-fw, 5′-GGAGAGAAGAAAAGTGACCTG-3′, Engrailed-RT-rv, 5′-GCCTCCTCTGCTCAGTCAAA-3′, HoxB9-RT-fw, 5′-TACAGTCAATTATCAGCCCCGA-3′, HoxB9-RT-rv, 5′-TGTAATGTTGGGGTCCCAGTTT-3′, NCAM-RT-fw, 5′-GCCATTCGTAAAGTGGAACCATA-3′, NCAM-RT-rv, 5′-CCAGTTTTGGAGCACTAGGTT-3′, N-tubulin-RT-fw, 5′-CCATTCTCTGGGTGGTGGCA-3′, N-tubulin-RT-rv, 5′CTGTGAGGTAGCGTCCATGAC-3′, a-actin-RT-fw, 5′-CTGACTGAACGTGGCTACTC-3′, a-actin-RT-rv, 5′-GTCAGCAATACCAGGGTACA-3′, ODC-RT-fw, 5′-GCAACTGATGCATGATATTAAAGAAC-3′, ODC-RT-rv, 5′-GAACTTTTATTTGTAAAACTGGTCAA-3′.

Adrenaline treatments

Approximately 91.6 mg of adrenaline powder (#E4125, Sigma, USA) was dissolved in 300 μL of acetic acid, and diluted by 0.1× SS up to a total 5 mL volume (100 mmol/L concentration in this period), and stored in a freezer as 500-μL aliquots (master solution). Master solutions were inactivated in a few weeks, so we always spent all of them in a few days. Five hundred microliters of master solution was diluted with 0.1× SS up to 50 mL volume and neutralized with 110 μL of 5N NaOH solution (1 mmol/L concentration in this period), and further dilutions were performed with 0.1× SS. Adrenaline treatments were performed in Poly-Hema (12% of poly 2-hydroxyethyl methacrylate solution, #18894, Polysciences, USA)-coated 40 mm diameter plastic plates using 3 mL of diluted adrenaline solutions under dark conditions. For Poly-Hema coating of plastic plate, 500 μL of 4% of Poly-Hema solution diluted by ethanol was spread on a plate, immediately removed, and then dried out. It seemed that adrenaline activity was time-dependently reduced at room temperature, so we started using it just before the designated time.

Cloning and mRNA synthesis and microinjection

Adrβ2 gene was cloned by PCR and ligated to pCS2 plasmid. The following primers were used for cloning from cDNA created from total RNA of stage 12 embryos; Adrβ2-fw, 5′-AATTGAATTCATGGAACGGTCG-3′ and Adrβ2-rv, 5′-AATTCTCGAGCCTATAATAAGCAA-3′. To generate synthetic mRNA *in vitro*, pCS2-Adrβ2 was linearized with NotI and transcribed with SP6 RNA polymerase by mMESSAGE mMACHINE kit (#AM1340, Ambion, USA). mRNA and MO microinjections into *Xenopus* embryo were performed at the 2-cell stage. The sequence of Adrβ2-MO was 5′-GGCTGGCGCTGACCGACCGTTCCAT-3′. For creating five-mismatch mRNA of *Adrβ2*, long-range PCR was performed for pCS2-Adrβ2 using KOD-plus NEO DNA polymerase (#KOD-401, Toyobo, Japan) and the following primers; 5′-ATGGAGCGGTCTGTTAGCGCTAGTCCTAA-3′ and 5′-GAATTCGAATCGATGGGATCCTGCAAAAAGAA-3′. The regions of mismatched nucleotides without changing amino acid sequence are shown in Figure 2A.

Western blot

Embryo lysate was prepared in PhosphoSafe Extraction Buffer (#71296, Novagen, USA), lipids removed by

extracting once with an equal volume of trichloroethylene (#208-02486, Wako, Japan), and proteins separated by 5–20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels (#197-15011, Wako, Japan). Western blots were performed using monoclonal rabbit antibodies against diphospho-ERK1/2 (1:2000, #4370, Cell Signaling, USA) and ERK1/2 (1:1000, #4695, Cell Signaling, USA).

Results

The receptor of adrenaline expresses from egg to tadpole

If adrenaline has functions at early embryogenesis, the receptor of adrenaline should be expressed at the early stage of embryos. It is actually reported that *Adrβ1* mRNA is expressed from oocyte (Devic *et al.* 1997) but the details of function of it and expressions of the other types of *β*-adrenergic receptors have never been investigated. Therefore, in order to examine the expression pattern of *Adrβ1* and *Adrβ2*, in embryos at various developmental stages, RT–PCR was carried out. Then, we found that high amounts of *Adrβ2* mRNA were expressed in all stages of *Xenopus* embryos, while *Adrβ1* was only maternally expressed (Fig. 1). In addition, we also checked the expression level of phenylethanolamine N-methyltransferase (PNMT), which is known as an enzyme to create adrenaline. Interestingly, PNMT started expression at stage 9 and continued to express until tadpole stages (Fig. 1), suggesting that adrenaline hormone must have some effect in early embryogenesis. We also tried to learn the expression area of *Adrβ2* and *PNMT*, but both genes seemed to express uniformly in all stages (data not shown).

Adrenaline has posteriorizing activity

In order to directly know the function of Adr*β*2, we designed Adr*β*2-MO, which specifically targeted the first 25 nucleotides of the open-reading frame (ORF) (Fig. 2A). We found that embryos lost tail structure by injection of Adr*β*2-MO (Fig. 2B,C), indicating that this receptor may have a role of posteriorization. If this loss-of-functional effect is true, gain-of-functional experiment of *β*-adrenergic signaling should have some sort of effects on A–P axis formation. Therefore, we cloned *Xenopus Adrβ2* gene by PCR and created its mRNA. As we expected, clear anterior defects were caused by both microinjection of *Adrβ2* mRNA (Fig. 2D). In order to rescue the effects of Adr*β*2-MO, we created a modified version of *Adrβ2* that had five mismatches in the first 25 of ORF region but still kept the same information of amino acids sequence (Fig. 2A). This modified mRNA (5mis-RNA) could induce posteriorization the same as the case of normal mRNA (Fig. 2D,E) and completely cancel the effect of Adr*β*2-MO (Fig. 2G) although normal mRNA did not do it efficiently (Fig 2F).

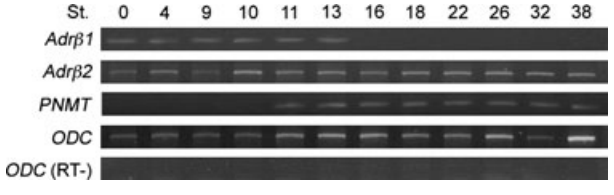

Fig. 1. Expressions of *Adrβ1*, *Adrβ2*, and *PNMT* mRNA during the *Xenopus* embryonic period. In all stages expression of *Adrβ2* were detected, while maternal or zygotical expressions were observed in *Adrβ1* and *PNMT* mRNAs, respectively. *ODC* was used for loading control.

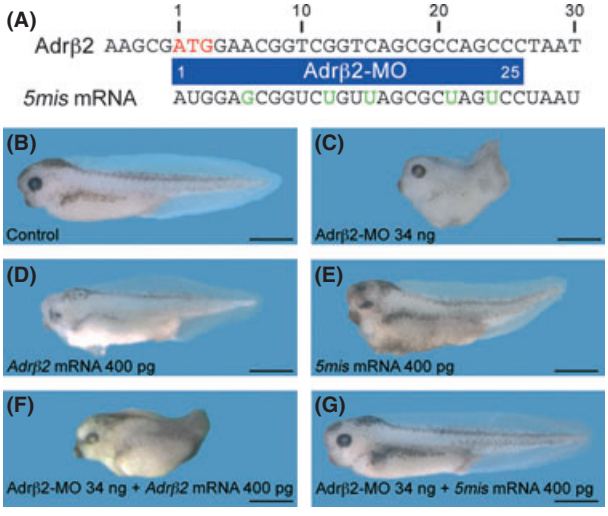

Fig. 2. Loss-of-functional effects by antisense morpholino oligomer for Adr*β*2 (Adr*β*2-MO) for *Xenopus* embryogenesis. (A) Experimental design using Adr*β*2-MO. Adr*β*2-MO was designed to target the translation initiation site (blue bar). This MO does theoretically not bind to artificially created mRNA containing five mismatched nucleotides (5mis) shown below. Red and green letters indicate start codon and changed nucleotides, respectively, without changing coding information. (B) Control embryo at stage 38. (C) Adr*β*2-MO injected embryos. 75% (6/8) of embryos lost tail structures. (D) *Adrβ2* mRNA injected embryos. 89.5% (17/19) of embryos lost eye structures. (E) *5mis* mRNA injected embryos.88.2% (15/17) of them lost eye structures. (F) Adr*β*2-MO and *Adrβ2* mRNA coinjected embryos. 9.5% (2/21) of embryos lost tail structures. (G) Adr*β*2-MO and *5mis* mRNA coinjected embryos. No embryo lost tail structures, and 53.6% (15/28) of embryos looked almost normal. Scale bars represent 1 mm.

To evaluate the effects of β-adrenergic signaling during development, we then used treatments of chemical reagent of adrenaline hormone on live *Xenopus* embryos (Fig. 3A). The treatments were performed from stage 8 (early gastrula) in dark conditions (Fig. 3A). The reason why we did not start treatments from earlier stages was because the effect of adrenaline reagent worked most effectively in this condition in our preliminary experiments. This reagent might be significantly inactivated in room temperature as time advances. Probably that is why the effects of adrenaline on early embryogenesis have never been reported. Interestingly, anterior structures such as eye and cement gland were clearly blocked in a dose-dependent manner of adrenaline hormone (Fig. 3B–G). Chemical reagent of adrenaline hormone should be dissolved in acetic acid, diluted to 1 mmol/L concentration, and then neutralized with NaOH. We confirmed that these neutralized solutions did not make any effects on early embryogenesis (Fig. 3B). Moreover, these effects by adrenaline hormone were completely rescued by microinjection of Adrβ2-MO (Fig. 3H) and amplified by *Adrβ2* mRNA (Fig. 3I). Then, we examined the effects of β-adrenergic signaling on molecular marker expressions of *Rx2a* and *HoxB9*, which were, respectively, anterior and posterior neural markers at tailbud stage embryo (Fig. 4). Compared to the expression pattern of control embryos (Fig. 4A,B), single injection of *Adrβ2* showed slight inhibition of *Rx2a* expression level at anterior region (Fig. 4C,D), and Adrβ2-MO clearly reduced the extent of *HoxB9* expression area at the posterior region (Fig. 4E,F). Expectedly, treatments of adrenaline hormone strongly reduced *Rx2a* expression but increased *HoxB9* expression (Fig. 4G,H), while these effects by chemical reagent were obviously rescued by microinjection of Adrβ2-MO (Fig. 4I,J). From these data, we confidently conclude that β-adrenergic signaling had a role of posteriorization activity for early development in *Xenopus*.

Anterior neural fate induced by BMP antagonist is not changed by β-adrenergic signaling

Chordin (Chd) is a large secreted protein expressed specifically in the BCNE center of the blastula stage embryo and is required for anterior neural formation (Kuroda *et al.* 2004; Ishibashi *et al.* 2008). In the cases of zebrafish and *Xenopus,* loss of Chordin reduces anterior neural formation (Schulte-Merker *et al.* 1997; Oelgeschläger *et al.* 2003). Chordin and Noggin double-homozygous mutants of mice have severe defects in the development of forebrain structure (Bachiller *et al.* 2000). In order to check whether β-adrenergic signaling is able to affect this anterior neuralization by

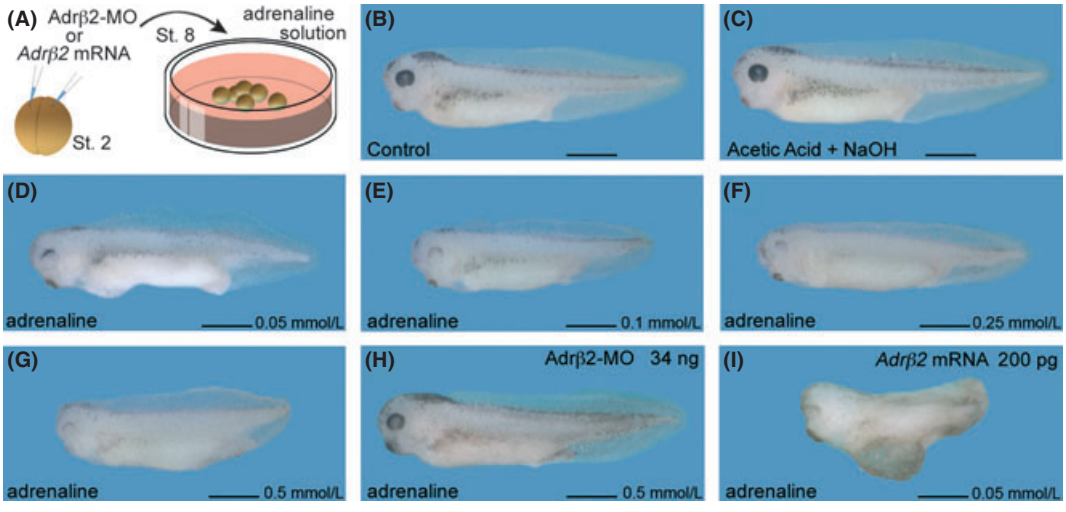

Fig. 3. Posteriorizing effects by adrenaline treatments. (A) Experimental procedures. Embryos injected with/without antisense morpholino oligomer for Adrβ2 (Adrβ2-MO) or with/without *Adrβ2* were cultured. Animal cap regions (ACs) were cut at stage 8 and then treated with various concentrations of adrenaline solutions (0.05–0.5 mmol/L). (B) Control embryos at stage 38. (C) Embryos treated with acetic acid solutions were neutralized with NaOH. All embryos (13/13) developed normally. (D–G) Embryos treated with 0.05, 0.1, 0.25, or 0.5 mmol/L of adrenaline solutions. 17.4% (4/23), 14.3% (4/28), 23.1% (6/26), or 27.6% (8/29) of embryos, lost eye structures. (H) Embryos treated with 0.5 mmol/L of adrenaline solution following microinjection of Adrβ2-MO. 45.6% (5/11) of embryos developed normally. Compared to G, it indicated that posteriorizing effects by adrenaline hormone were clearly rescued by Adrβ2-MO. (I) Embryos treated with 0.05 mmol/L of adrenaline solution following microinjection of *Adrβ2* mRNA. 80.0% (8/10) lost eye structures. Compared to D, it indicated that posteriorizing effects by adrenaline hormone were clearly amplified by *Adrβ2* mRNA. Scale bars represent 1 mm.

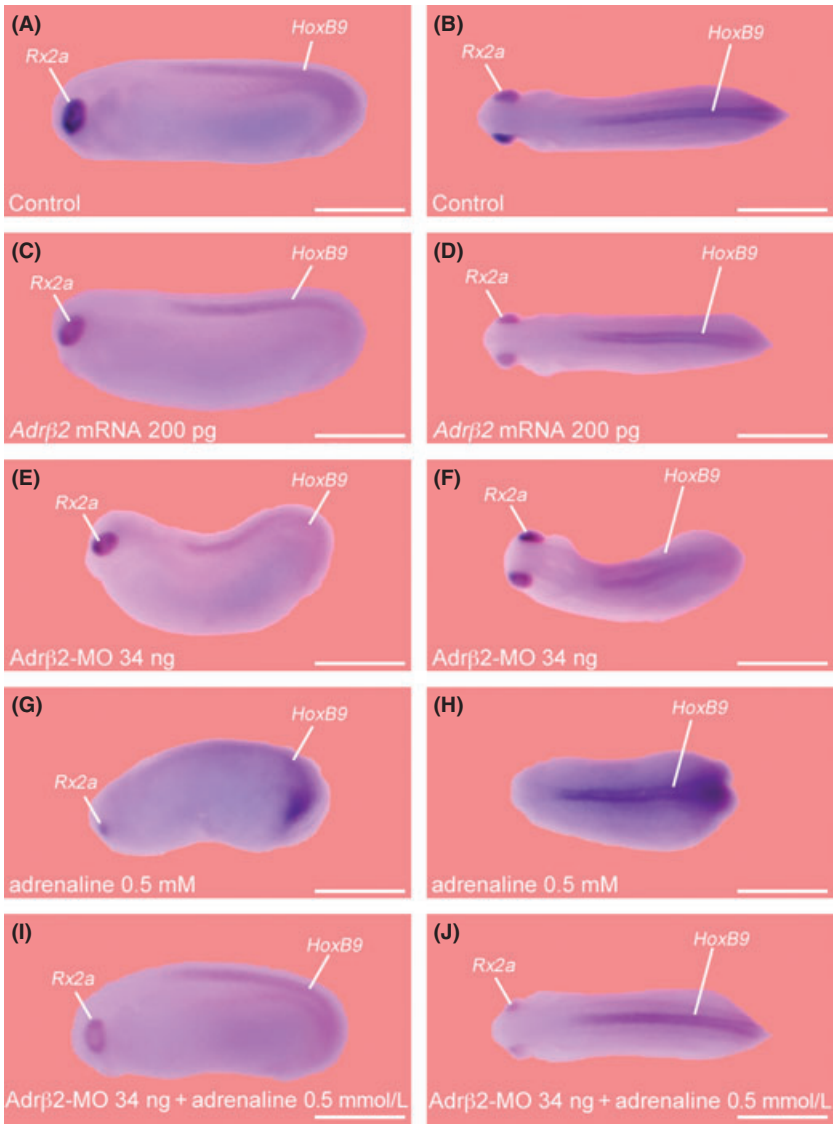

Fig. 4. The effects of β-adrenergic signaling on anterior and posterior neural marker expressions. (A, C, E, G, I) Lateral views of embryos. (B, D, F, H, J) Dorsal views of embryos. (A, B) Control embryos. (C, D) Embryos injected with *Adrβ2* mRNA. (E, F) Embryos injected with antisense morpholino oligomer for Adrβ2 (Adrβ2-MO). (G, H) Embryos treated with adrenaline solutions. (I, J) Embryos treated with adrenaline solutions following microinjection of Adrβ2-MO at 2-cell stage. Scale bars represent 0.5 mm.

BMP antagonism, we then used a presumptive ectoderm region, which is generally called the animal cap region (AC). AC were cut from blastula stage embryos that were injected with mRNAs and/or MO at the 2-cell stage and cultured with/without adrenaline hormone until stage 26 to examine anterior neural markers *Six3* and *Rx2a*, mid-hindbrain boundary marker *En2*, posterior neural markers *HoxB9*, pan-neural markers *NCAM* and *N-tubulin*, mesoderm marker *α-actin*, and loading control *ODC* (Fig. 5A). The expression of both anterior neural and pan-neural markers induced by *Chd* was not blocked by both gain-of-functional way of β-adrenergic signaling activity (Fig. 5B) and loss-of-functional way (Fig. 5C). These results indicated that β-adrenergic signaling worked through mesoderm and indirectly changed anterior neural fate in embryos, so some mesodermal signals should be taken into account. It has been reported that Adrβ2 stimulation can activate extracellular signal-regulated kinase, ERK (Fig. 5D, Sivamani *et al.* 2007; Yang *et al.* 2010). Therefore, we next focused on phosphorylation of ERK. We simply put early stage of *Xenopus* embryos into adrenaline solutions and cultured, resulting in the phosphorylation level of ERK being remarkably increased when β-adrenergic signaling was upregulated (Fig. 5E).

Late effect of β*-adrenergic signaling causes gastrointestinal malformation*

Adrenergic neurotransmitters, which is also called norepinephrine or noradrenaline, is involved in the formation of regularly-structured visceral morphogenesis of *Xenopus* embryos (Toyoizumi *et al.* 1997). In our

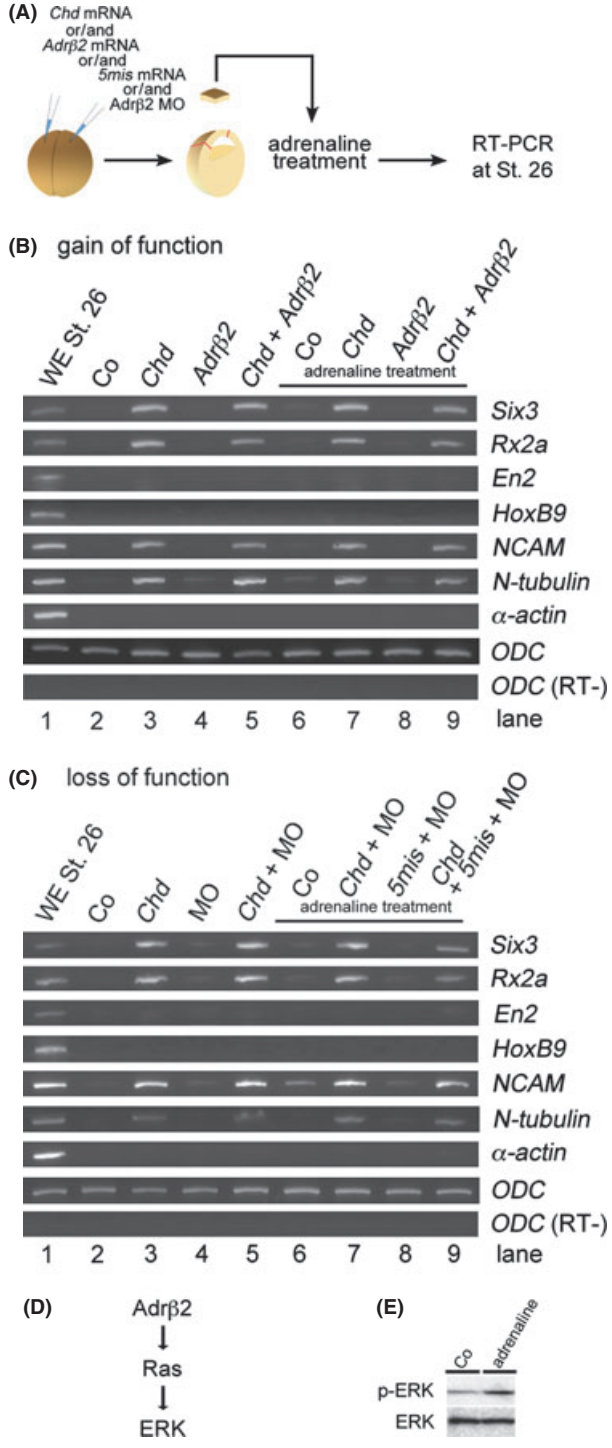

Fig. 5. The effects of β-adrenergic signaling on mesoderm-independent neural marker expressions. (A) The experimental procedure for B and C. Animal cap regions (ACs) were cut from embryos injected with/without mRNAs and/or Adrβ2-MO, cultured with/without 1 μM of adrenaline solution until stage 26, and used for reverse transcription–polymerase chain reaction (RT–PCR) analyses. More than 0.01 mmol/L of adrenaline solution killed AC cells for unfathomable reasons. (B, C) Inhibition of anterior neural marker expression by up- or downregulation of β-adrenergic signaling. Anterior neural markers, *Six3* and *Rx2a*, mid-hindbrain boundary marker, *En2*, posterior neural (spinal cord) marker, *HoxB9*, and pan-neural markers, *NCAM* and *N-tubulin*, were not affected in both cases. α-*actin* and ODC were respectively used for showing no contamination of mesoderm and loading control. (D) Downstream model of Adrβ2. (E) Western blotting analysis of phospho-ERK in adrenaline treated embryos. Embryos were treated from 2-cell stage with 0.5 mmol/L of adrenaline solution and fixed at stage 26.

experiments shown above, it was very difficult to evaluate gastrointestinal formation because A–P effects were too strong, and adrenaline hormone should be completely inactivated before reaching the tadpole stage. Therefore, we tried to start treatments of adrenaline hormone since stage 30, which is thought to be the initiation period of the morphogenetic movement of gastrointestinal formation in *Xenopus* development, and fixed embryos at stage 45. Compared to control embryos (Fig. 6A–D), neutralized solution, which was the same solution used in (Fig. 3C), did not have any effect (Fig. 6E–F). However, embryos treated with 0.5 mmol/L adrenaline hormone still keep all gastrointestinal organs, but all of them, especially liver and small intestine, were not well developed (Fig. 6I–L). In the case of 1 mmol/L, although only a few organs such as lung and pancreas were observed, separation of small and large intestine and bile duct formation were not detected at all (Fig. 6M–P). Interestingly, in these late-stage treatments, tail formation was obviously shortened and shrunken (Fig. 6 M,N). Heartbeat is started just after stage 40, and the bloodstream carries many secreted molecules and hormones away. Probably, many tissues and organs may already require supply of adrenaline from the bloodstream for growth. Gastrointestinal malformation can easily occur if developmental timing is disordered by rapamycin reagents, which is target of rapamycin (TOR) kinase inhibitor (Moriyama *et al.* 2011), so it is quite possible that a similar mechanism is working in the case of adrenaline treatment on late stage embryos.

Discussion

In this work, we have investigated the functions of β-adrenergic signaling on *Xenopus* early development and defined that it has a posteriorizing activity via mesodermal signal in the early stage. The neural ectoderm is patterned along its A–P axis. This patterning is initiated by posteriorizing signals derived from prospective or definitive mesendodermal tissues (Nieuwkoop

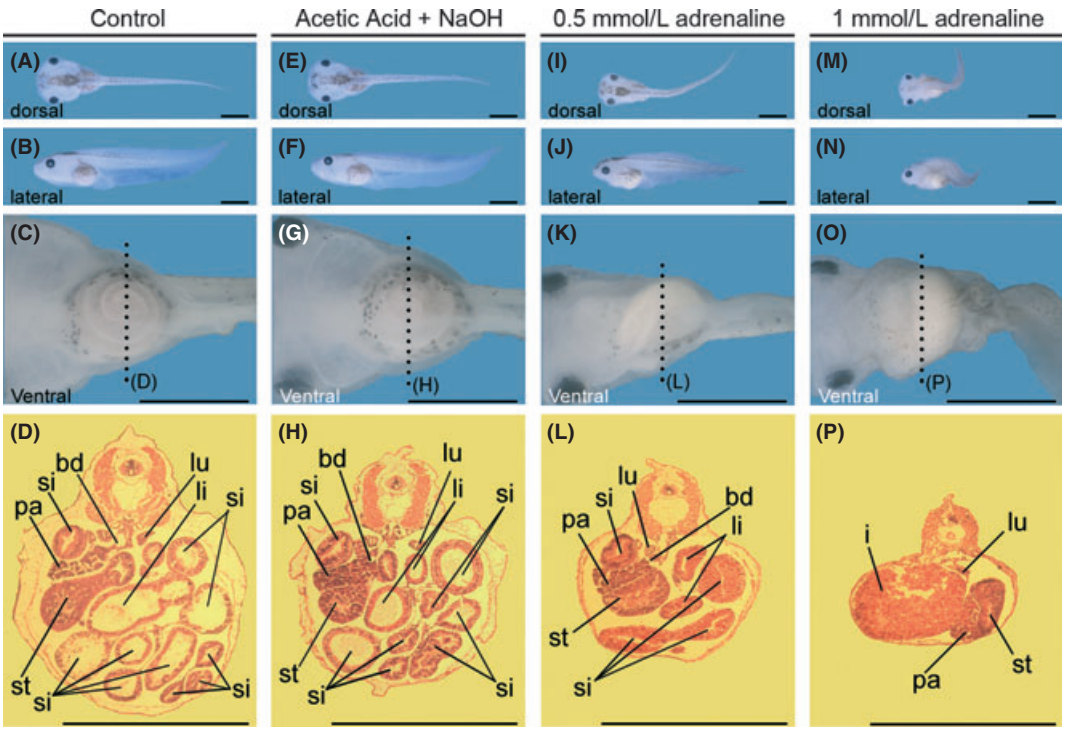

Fig. 6. The late effect of β-adrenergic signaling on *Xenopus* embryogenesis. (A, E, I, M) dorsal, (B, F, J, N) lateral, and (C, G, K, O) ventral and enlarged view of embryos. (D, H, L, P) Histology of gastrointestinal regions. Sectioned sites were indicated in C, G, K, O by dotted lines. (A–D) Control embryo. (E–H) 100% (3/3) of embryos treated with acetic acid solution neutralized with NaOH. (I–L) 100% (3/3) embryos treated with 0.5 mmol/L of adrenaline solutions. (M–P) 100% (4/4) of embryos treated with 1 mmol/L of adrenaline solutions. bd, bile duct; i, intestine; li, large intestine; lu, lungs; pa, pancreas; si, small intestine; st, stomach. Scale bars represent 1 mm.

1952; Toivonen & Saxen 1968). Three candidate posteriorizing signals have been suggested: retinoic acid (RA; Durston *et al.* 1989; Sive *et al.* 1990; Conlon 1995; Blumberg *et al.* 1997), fibroblast growth factors (Fgfs; Kengaku & Okamoto 1993;Cox & Hemmati-Brivanlou 1995; Lamb & Harland 1995; Koshida *et al.* 1998), and Wnts (Kelly *et al.* 1995; McGrew *et al.* 1995; Fekany-Lee *et al.* 2000; Kazanskaya *et al.* 2000; Kiecker & Niehrs 2001; Yamaguchi 2001). Although it is likely that factors in all three families participate in this process, the precise role of each in the temporal and spatial aspects of neural patterning as well as the molecular consequences of their action have not been fully clarified, but posteriorizing activity by β-adrenergic signaling is highly possible to be related with at least one of these three candidate posteriorizing signals.

Mechanism of posteriorization by β*-adrenergic signaling*

Anterior neural marker expressions induced by Chd were not affected by both upregulation and downregulation of β-adrenergic signaling, but interestingly phosphorylation level of ERK was strikingly increased by adrenaline treatment (Fig. 5). We think that it is quite reasonable that strong ventralization should occur if β-adrenergic signaling blocks BMP signaling activity, but we did not observe it. This strongly suggests that β-adrenergic signaling is not a simple posteriorizing factor on neuroectoderm and has other activity to affect the mesodermal region. It is likely to be caused by upregulation of FGF signaling because phosphorylation of ERK can also be induced by FGF signaling (Kuroda *et al.* 2005). FGF is able to change the fate of ectoderm into mesoderm (Kimelman & Kirschner 1987), but mesodermal marker was not detected in (Fig. 5B), and elongation or swelling phenotypes that are usually observed in mesoderm-induced AC explants were not observed either (data not shown). This means that posteriorizing activity of β-adrenergic signaling can work on the basis of presence in mesoderm.

Crosslink of β*-adrenergic and FGF signaling*

The catecholamines epinephrine (adrenaline) and norepinephrine (noradrenaline) are agonists for a family of G-protein coupled receptors (GPCRs) known as adrenergic receptors. There are three subfamilies of adrenergic receptors: α1, α2 and β, and the β

subfamily contains three subtypes: β1, β2, and β3 located predominantly in heart, lung and adipose tissue, respectively (Ma & Huang 2002). Adrenergic receptors are GPCRs that link to trimeric G-protein. G-proteins are typically composed of α, β and γ subunits. Each subunit exists in multiple isotypes with differential specificity for effector signaling. Specificity of response to catecholamines is mediated in part by the Gα subunits, but the Gβ-Gγ dimers are also involved in regulation. β-adrenergic receptors favor interaction with heterotrimeric G-proteins that contain the Gα-s and Gα-i subunit. The Gα-s subunits activate various isoforms of adenylate (adenylyl) cyclases. Consequently, β-adrenergic receptors typically elevate the level of cyclic AMP (cAMP) an important mediator of cell signaling. Both Gα-s and Gα-i have been linked to the stimulation of Src family tyrosine kinases (Huang *et al.* 2004). Src tyrosine kinase is a major downstream factor of FGF receptor, and Src tyrosine kinase activated by FGF receptor contributes to certain FGF-induced biological responses via the Raf1-MEK-ERK pathways, and it has strong posteriorizing activity (Kuroda *et al.* 2004). These suggest that the A–P patterning in embryonic body plan may be regulated by activation of Raf1-MEK-ERK via upstream crosstalk between β-adrenergic and FGF signaling in the cytoplasmic region.

Acknowledgments

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