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Axis elongation during *Xenopus* tail-bud stage is regulated by GABA expressed in the anterior-to-mid neural tube

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ABSTRACT The receptors of gamma-aminobutyric acid (GABA), which is a well-known neurotransmitter, are expressed in the anterior-to-mid neural tube at an early stage of *Xenopus* development, but there has been no report on the role of GABA in the presumptive central nervous system. Therefore, we tried to reveal the function of GABA for *Xenopus* early embryogenesis. We first confirmed that the region expressing a gene encoding glutamate decarboxylase 1 (*gad1*), which is an enzyme that catalyzes the decarboxylation of L-glutamate to GABA, overlapped with that of several genes encoding GABA receptors (*gabr*) in the neural tube. Metabolome analysis of culture medium of dorsal tail-bud explants containing the neural tube region of tail-bud stage embryos also revealed that GABA was expressed at this stage. Then, we examined the treatment of pentylenetetrazole (PTZ) and picrotoxin (PTX), which are known as inhibitors of GABA receptors (GABA-R), on the early stages of *Xenopus* embryogenesis, and found that axis elongation in the tail-bud was inhibited by both treatments, and these phenotypic effects were rescued by co-treatment with GABA. Moreover, our spatial- and temporal-specific inhibitor treatments revealed that the *gabr*- and *gad1*-overlapped region, which presents at the anterior-to-mid neural tube during the tail-bud stages, was much more sensitive to PTZ and thus caused severe inhibition of axis elongation. Taken together, our results indicate that the small ligand molecule GABA functions as a regulator to induce the axis elongation event in the tail-bud during early embryogenesis via direct stimulation of the neural tube and indirect stimulation of the surrounding area.

KEY WORDS: *GABA, Xenopus, neural tube, convergent extension, pentylenetetrazole*

Introduction

In the field of developmental biology, various secreted proteins have been reported as regulators of early embryogenesis. However, there are few studies about the roles of small ligand molecules below 500 Da. Retinoic acid, which has 300 Da, is reported to be a strong posteriorizing factor in vertebrate early development (Blumberg *et al.*, 1997). Adrenaline, 183 Da, also functions as a posteriorizing factor (Mori *et al.*, 2013), whereas similar molecules noradrenaline, 169 Da, and monoamine neurotransmitter serotonin, 176 Da, both work as regulators for left right axis formation (Toyoizumi *et al.*, 1997; Fukumoto *et al.*, 2005). Although many small ligand molecules, which are not directly coded in genes, such as neurotransmitters and hormones, exist in adults, they have not been reported well as regulators of early embryogenesis. In early embryogenesis, the relationship between protein agonists and antagonists has been studied very well, and major experimental methods have focused on DNA- and mRNA-related works. Therefore, screening methods to find small ligand regulators may not be well established in this field.

Abbreviations used in this paper: GABA, gamma-aminobutyric acid; GABA-R, GABA receptor; GAD, glutamate decarboxylase; PTX, picrotoxin; PTZ, pentylenetetrazole.

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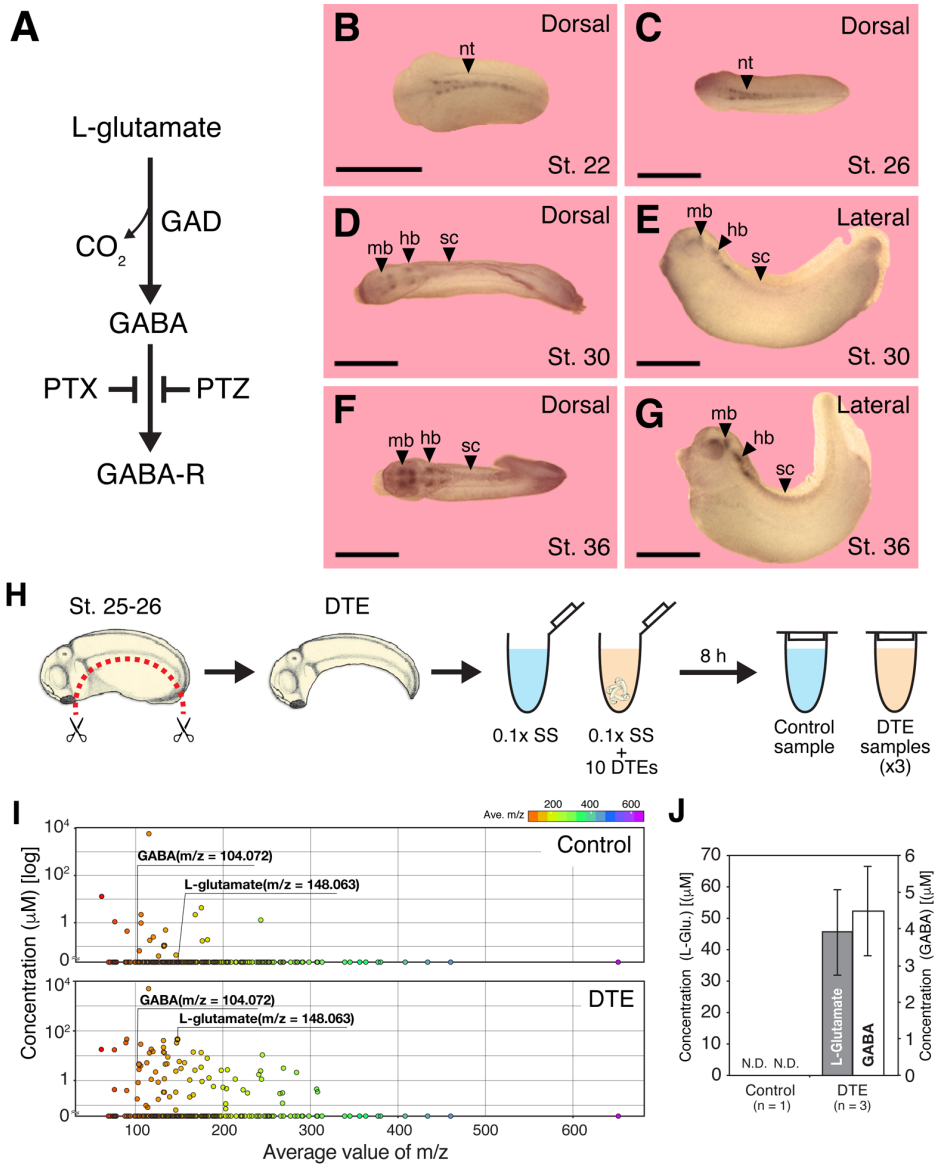

Fig. 1. *gad1* and GABA are expressed in the tail-bud stages. (A) *Schematic representation of the GABA pathway. GABA, gamma-aminobutyric acid; GABA-R, GABA receptors; GAD, glutamic acid decarboxylase; PTZ, pentylenetetrazole; PTX, picrotoxin.* **(B-G)** *Whole-mount* in situ *hybridization analysis of* gad1 *of embryos at stages 22 (B), 26 (C), 30 (D, E), and 36 (F, G). nt, neural tube; sc, spinal cord; mb, midbrain; hb, hindbrain. Scale bars represent 1 mm.* **(H)** *Schematic diagram of the sampling method for metabolome analysis. Yolk-rich and most ventral regions were removed from embryos at stages 25 26. The remaining area was called the dorsal tail-bud explant (DTE). 10 DTEs were cultured in 0.1x Steinberg's solution for 8 h, and the supernatant was used for metabolome analysis. SS, Steinberg's solution.* **(I)** *Concentration of cations detected by CE-TOF-MS analysis in the control sample (upper; n=1) and DTEs (bottom; n=3, obtained from 2 female frogs). The m/z represents mass divided by charge number.* **(J)** *Concentrations of L-glutamate (m/z = 148.063) and GABA (m/z = 104.072) in the control and DTEs. Data represent mean±S.D. N.D., not detected. Data sets are the same as those in Fig. 1I.*

Gamma-aminobutyric acid, GABA, which has 103 Da, is one of the most well-known neurotransmitters. In vertebrates, GABA acts at inhibitory synapses in the brain by binding to specific transmembrane receptors, causing the opening of ion channels to allow the flow of either negatively charged chloride ions into the cell or positively charged potassium ions out of the cell (Lorenz-Guertin *et al.*, 2017). The role of GABA is also reported to be a developmental switch between neonatal and adult stages on neural cells by changing the concentration of chloride inside cells (Li and Xu, 2008), whereas amphibian oocyte maturation is required for activation of GABA-R (Toranzo *et al.*, 2008). These data suggest that GABA functions as not only a neurotransmitter but also as a ligand molecule to induce other biological events. Interestingly, temporal expression patterns of genes coding GABA type A receptors ($GABA_A$-R) and type B receptors ($GABA_B$-R) during the tail-bud stages were detected in *Xenopus* embryos (Kaeser *et al.*, 2011). Therefore, it should be very reasonable to expect that GABA has some activities for a developmental event in early embryonic stages, especially in the tail-bud stages.

In this study, we showed clear evidence that GABA exists in embryonic stages and introduced the function of GABA by treatment with the GABA signal inhibitors such as pentylenetetrazole (PTZ) and picrotoxin (PTX).

Results

gad1 *is expressed in the neural tube during tail-bud stages*

L-glutamate is important for GABA synthesis, and glutamate decarboxylase (GAD) is known to catalyzes a decarboxylation reaction to produce GABA from L-glutamate (Fig. 1A). If GABA is expressed and functions in early embryonic stages, *gad1* should be expressed in embryos. Transcriptome analyses for *Xenopus tropicalis* embryos show that expression of *gad1* starts around stage 20 and continues until stage 40 (Owens *et al.*, 2016). Therefore, in order to examine the expression patterns of *gad1*, *in situ* hybridization was carried out in the tail-bud embryos. *gad1* expressions during the early tail-bud stages (stages 22 and 26) were detected in part of the neural tube, which would become the future posterior brain and anterior-to-mid spinal cord (Fig. 1 B,C). Interestingly, in both stages the expressions of *gad1* were relatively weak in the dorsal midline region of the neural tube and were intermittent (on-again-off-again) at all expressed area (Fig. 1 B,C). In the mid tail-bud stage (stage 30) *gad1* was expressed in the midbrain, hindbrain, and anterior spinal cord (Fig. 1 D,E), and these expression patterns were still maintained at the late tail-bud stage (stage 36; Fig. 1 F,G). The intermittent expression pattern became more obvious on the anterior side, and no expression was detected in the anterior

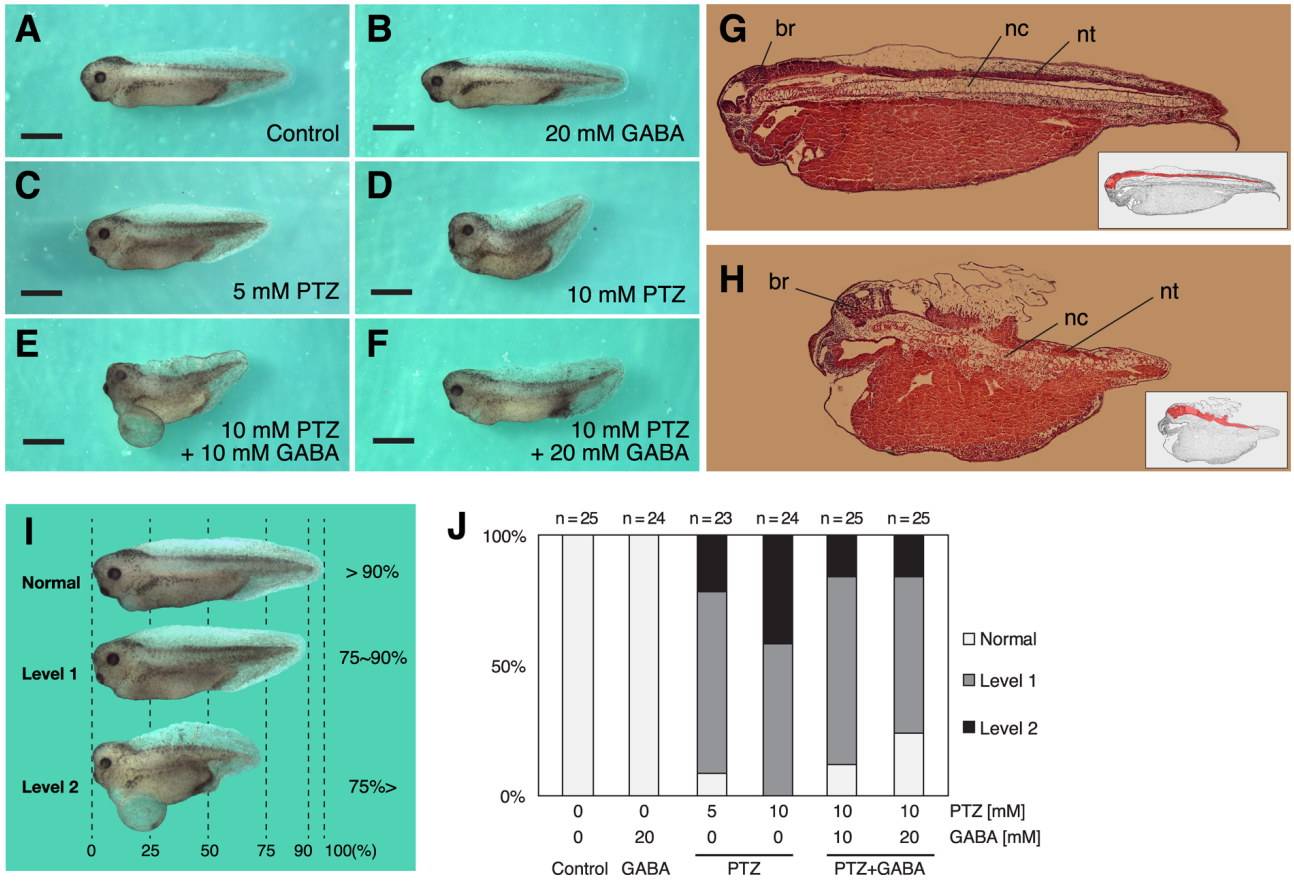

Fig. 2. Elongation of body axis is inhibited by pentylenetetrazole (PTZ) treatment. (A-F) *Results of PTZ treatment assay from stages 8 - 38. Scale bars represent 1 mm.* **(A)** *Control embryo.* **(B)** *Embryo treated with 20 mM GABA.* **(C)** *5 mM PTZ.* **(D)** *10 mM PTZ.* **(E)** *10 mM PTZ and 10 mM GABA.* **(F)** *10 mM PTZ and 20 mM GABA.* **(G,H)** *Sagittal histological section of control embryo at stage 38 (G) and embryo treated with 10 mM PTZ (H). A red region in a right-bottom small panel indicates the neural region in G and H. br, brain; nc, notochord; nt, neural tube.* **(I)** *The body-length index table to quantify inhibitory action in treated embryos. We categorized embryos into 3 groups: embryos had >90% body length (Normal), 75-90% body length (Level 1), and <75% body length (Level 2) referring to the average length of control embryos.* **(J)** *Quantification of inhibitory action by the PTZ treatment. See (I) for the degree of defects. Sample number is indicated at the upper side of each bar graph.*

dorsal midline region (Fig. 1 D,F). These data and a past report showing that genes coding GABA-R (*gabr*) are expressed at the neural tube in tail-bud embryos (Kaeser *et al*., 2011) suggest that the ligand molecule for GABA-R, GABA, is active during these stages of *Xenopus* embryogenesis.

GABA is detected in tail-bud embryos by metabolome analysis

Even if both synthase (or synthetase) and receptors for a ligand molecule are expressed, this does not guarantee expression of the ligand molecule itself. Therefore, in order to monitor the expression of GABA, we performed a metabolome analysis on the supernatant from dorsal tail-bud explants (DTEs) culture medium (Fig. 1H). The reason why ventral part was removed is because yolk-rich region interferes with metabolite analysis. Metabolomics is the comprehensive assessment of endogenous metabolites and systematic identification and quantification of metabolites from a biological sample. We found that both GABA and its precursor molecule L-glutamate were detected from DTEs cultured solution, whereas no signal for either molecule was found in the control solution, 0.1x Steinberg solution (SS) (Fig. 1 I,J). Taking these results together, we assume that GABA certainly has a certain role in the tail-bud of *Xenopus laevis*.

Axis elongation is inhibited by pentylenetetrazole treatment

Gain-of-function experiments of GABA are easy to design because GABA is a very common commercial reagent. Therefore, we chose the GABA treatment of embryos as the first functional analysis of GABA on early embryogenesis. However, we could not detect any difference between the control and treated embryos in the case of less than 100 mM concentrations of GABA (Fig. 2 A,B; Supplementary Fig. S1 A-C). Interestingly, in the case of very high concentrations such as 200 mM, all embryos treated with GABA showed shrinkage of the larval fin, but the same phenotypic effects were observed when embryos were treated with 200 mM of alanine (89 Da), glycine (75 Da), or D-glucose (180 Da; Supplementary Fig. S1 D-G). Thus, we conclude that these phenotypic effects in larval fins induced by high concentration of GABA were just caused by the effects of a hyperosmolar solution.

The fact that treatments of GABA did not have any effect on early embryogenesis may indicate that GABA does not have a role in embryogenesis or that the effect of GABA reaches an almost maximum level in embryos. Therefore, we thought that a loss-of-function experiment for GABA was still worth doing to know the role of GABA. One of the simplest ways to do loss-of-function experiment is to use dominant-negative GABA-R or an antisense morpholino oligo

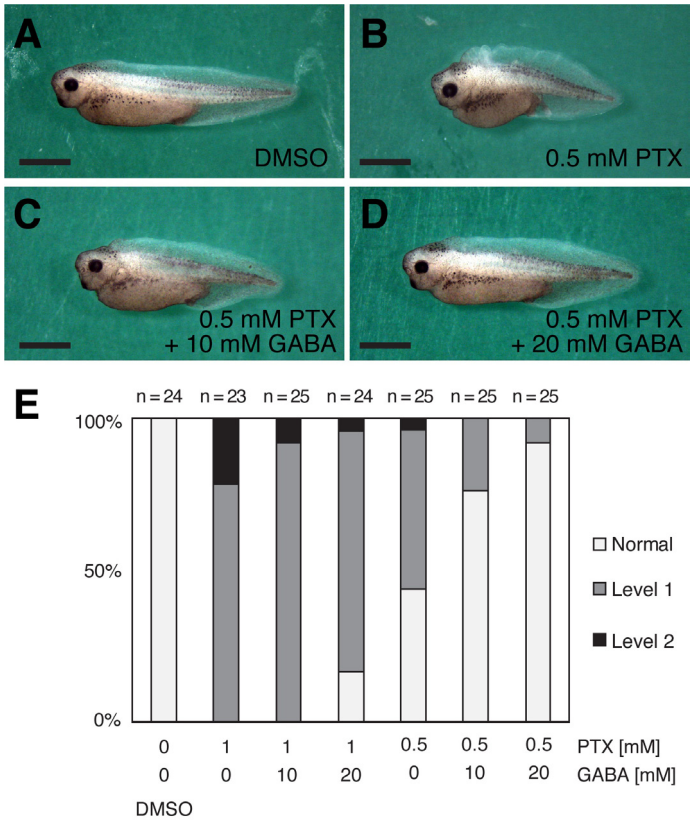

Fig. 3. Elongation of body axis is inhibited by picrotoxin (PTX) treatment. (A-D) *Results of PTX treatment assay from stages 8838. Scale bars represent 1 mm.* **(A)** *Embryo treated with 1% DMSO.* **(B)** *0.5 mM PTX.* **(C)** *0.5 mM PTX and 10 mM GABA.* **(D)** *0.5 mM PTX and 20 mM GABA.* **(E)** *Quantification of the PTX treatment. See Fig. 2I for the degree of defects. Sample number is indicated at the upper side of each bar graph.*

for inhibition of translation of GABA-R. However, it is very difficult to design a loss-of-function experiment targeting GABA-R because there are mainly two classes of GABA receptors such as $GABA_A$-R and $GABA_B$-R: the $GABA_A$-R class has various subunits to create ligand-gated ion channels (also known as ionotropic receptors), whereas the $GABA_B$-R class has a G protein-coupled mechanism (also called metabotropic receptors). Similarly, for GABA-R, it is difficult to make morpholino oligos for *gad* because at least three multiple genes are found as *gad* in the database. Another idea for a loss-of-function experiment was to use PTZ (138 Da), which is a typical inhibitor for the GABA signal. PTZ, also known as metrazol, pentetrazol (INN), pentamethylenetetrazol, Corazol, or Cardiazol, is a drug used as a circulatory and respiratory stimulant in the field of medicine, and its inhibitory action is caused by binding to the PTX-binding site on $GABA_A$-R (Squires *et al.*, 1984).

Remarkably, when embryos were treated with 5 or 10 mM of PTZ, axis elongation was inhibited (Fig. 2 C,D), and this short-axis phenotype was mildly rescued by co-treatment with 10 mM of GABA (Fig. 2E) and strongly rescued with 20 mM of GABA (Fig. 2F). Although simple addition of GABA did not lead to morphological changes, it acted on the GABA signal-inhibited embryos, indicating that GABA might have a role as an elongation signal in embryos. To visualize the morphological changes induced by PTZ, histological sections were created for control and PTZ-treated embryos at stage 38 (Fig. 2 G,H). Compared to control embryos (Fig. 2G), severe malformation of the neural tube was observed in PTZ-treated embryos (Fig. 2H). In addition, the thickness of the notochord was expanded, and the border between the notochord and surrounding tissues was ambiguous in PTZ-treated embryos (Fig. 2H). Both tissues did not have GABA-R, so this result indicates that some intermediate effect downstream of the GABA signaling-activated neural tube should be concerned. Interestingly, a morphologically atypical shape was observed in the dorsal epidermis region in PTZ-treated embryos probably because of swelling due to the short-axis phenotype (Fig. 2H). We further designed a body-length index table in order to quantify the inhibitory action in treated embryos (Fig. 2I). Compared to the average length of control embryos, treated embryos having a 90-100%, 75-90%, or less than 75% length were respectively evaluated as normal, level 1, or level 2 (Fig. 2I). According to this table, we summarized these results from treatments of PTZ with/without GABA, and it clearly showed the dose-dependent manners of PTZ and GABA (Fig. 2J).

The effects of picrotoxin are the same as pentylenetetrazole

As shown in Fig. 2, PTZ had the activity of inducing a short axis phenotype when added, but we should consider the possibility of a GABA-R-nonspecific effect by PTZ. To examine whether PTZ works specifically or not on GABA-R, we also checked the activity of another prototypic antagonist of $GABA_A$-R, PTX (Olsen, 2006). First, PTX was dissolved in DMSO and then diluted with 0.1x SS because PTX is insoluble in water, although PTZ is soluble. First, we confirmed that no effect was caused by treatments with 1% DMSO in 0.1x SS, which was the maximum DMSO concentration as the solution containing PTX in our designed experiments in this work (Fig. 3 A,E). This is consistent with previous reports, which also found no toxicity of 1% DMSO for early *Xenopus* embryogenesis (Moriyama *et al.*, 2011). Then, we tested embryos with 0.5 or 1 mM of PTX, resulting in short axis phenotypes similar those by treatment with PTZ, which were induced in a dose-dependent manner (Fig. 3 B,E). In particular, the effect of 1 mM of PTX was almost the same as 10 mM of PTZ (Fig. 2J; Fig. 3E), meaning the activity of PTX for the inhibitory action on $GABA_A$-R may be almost 10-times stronger than PTZ. These phenotypic effects induced by PTX treatment were rescued by addition of GABA (Fig. 3 C-E). Here we had two options to block the GABA signal: PTZ and PTX. We decided to keep using PTZ for additional experiments because PTZ is soluble and more common than PTX as an inhibitor of GABA. Taken together, our inhibitor treatments implied that GABA activity is required for axis elongation in *Xenopus* embryogenesis.

Elongation of the embryonic axis is induced by the GABA signal in the neural tube at the tail-bud stage

Both PTZ and PTX are thought to act on $GABA_A$-R, which has several subunits such as α1, α2, α3, α5, α6, ρ1, ρ2, and ρ3, and most of these subunits are expressed in the neural tube in the tail-bud stage (Kaeser *et al.*, 2011). The expression area and timing were widely overlapped with the *gad1* expression pattern (Fig. 1B(G), suggesting that the inhibitory action of GABA inhibitors might be effective in the tail-bud stages and overlapped area such as the neural tube including the presumptive posterior brain and anterior-to-mid spinal cord. In order to understand the spatiotemporal activation of the GABA signal in embryos, we first designed the time-specific PTZ treatments for embryos, meaning that the PTZ

treatments were performed during stages 3-13 (before neurula, Fig. 4B), 13-20 (mainly neurula, Fig. 4C), 20-28 (early-to-mid tail-bud, Fig. 4D), or 28-38 (mid-to-late tail-bud, Fig. 4E) in addition to 3-38 (Fig. 4F) and control (Fig. 4A). As the results, very strong effects were observed when embryos were treated with PTZ in stages 20-28 (Fig. 4 D,G) and 28-38 (Fig. 4 E,G), which were similar to the effects in stages 3-38 (Fig. 4 F,G), and no effects were observed in stages 3-13 (Fig. 4 B,G) and 13-20 (Fig. 4 C,G), which was the same as control embryos (Fig. 4 A,G). These results indicate that the sensitivity in reaction to PTZ appears around initiation of the *gad1* expression in embryos. In order to examine site-specificity of GABA activation, we next performed the PTZ treatment on dissected embryos (Supplementary Fig. S2). When embryos reached stage 26, we cut them at the anterior, mid, or posterior position (Supplementary Fig. S2, A-C) and cultured the dissected embryos with/without PTZ until stage 38 (Supplementary Fig. S2, D-I). The reason why dissected embryos were prepared was because, in addition to increase the permeability of reagents, the effect of PTZ for the *gabr*- and *gad1*-overlapped region (GGR, shown in Supplementary Fig. S2A, indicated in red) might be observed more clearly in dissected embryos. Remarkably, the short-axis phenotypic effects induced by PTZ were drastically apparent in posterior explants when cut at the anterior site (Supplementary Fig. S2 A,D,G). In this case, most of the expression of GGR was included in the posterior parts, so it is very reasonable to assume that PTZ functions well for the posterior parts. Similar effects of the PTZ treatment were also observed in the posterior parts when embryos were cut at the mid site (Supplementary Fig. S2 B,E,H) and anterior parts when embryos were cut at the posterior site (Supplementary Fig. S2 C,F,I). These results show that PTZ can work site-specifically in the tail-bud embryo in *Xenopus*.

To summarize, the results of spatiotemporal treatments of GABA inhibitors suggested that GABA strongly functions in the GGR of the neural tube to elongate the embryonic body axis during the tail-bud stages.

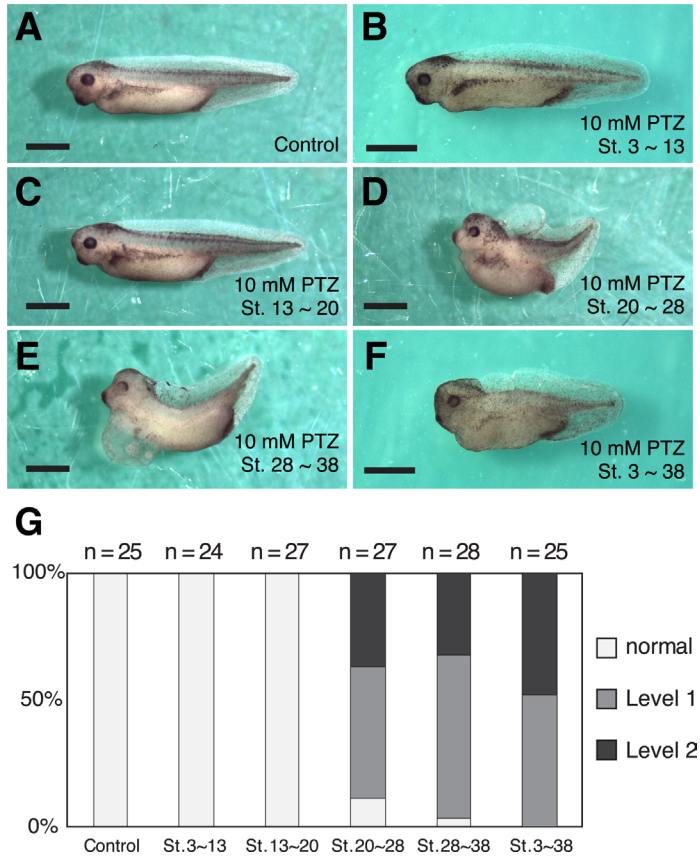

Fig. 4. Pentylenetetrazole (PTZ) treatment is effective at the tail-bud stage. (A-F) *Results of PTZ treatment assay in different time windows: control (A), from stages 3-13 (B), from stages 13-20 (C), from stages 20-28 (D), from stages 28-38 (E), and from stages 3-38 (F). Scale bars represent 1 mm.* **(G)** *Quantification of the PTZ treatment. See Fig. 2I for the degree of defects. Sample number is indicated at the upper side of each bar graph.*

Discussion

In this study, we investigated the role of GABA by using chemical reagents PTZ and PTX on *Xenopus* early development. We first found that the expression pattern of *gad1* was overlapped with the expression area of genes coding the GABA-R family (Fig. 1). Then, it was revealed that both PTZ and PTX induced the same effects such as a short-axis phenotype (Fig. 2, 3), and that the effect of PTZ was significantly prominent for the explants with GGR at the tail-bud stage (Fig. 4, Supplementary Fig. S2).

In developmental biology, the first step of embryonic elongation in vertebrates is caused by convergent extension of ectodermal and mesodermal tissues following gastrulation movement driven from the directional migration of leading-edge mesoderm (Hara *et al.*, 2013; Shindo, 2017). This elongation mechanism in *Xenopus laevis* is easily observed by the method named the Keller explant, in which explants are sandwiched between two rectangles of dorsal mesendoderm and ectoderm from an embryo at early gastrula stage (Keller *et al.*, 1989; Doniach, 1993; Saint-Jeannet *et al.*, 1994; Kuroda *et al.*, 2004). In these explants, elongation was observed when control embryos were from stages 10 20. No elongation was observed after that (Kuroda *et al.*, 2004), but normal embryos still kept elongating after stage 20. In other words, convergent extension is actually the first event and probably worked as a kind of driving force to elongate embryos, but this is far from being a sufficient explanation about why embryos are elongated from a 1.2-mm diameter egg (stages 1-10) to a 5-mm long embryo (stage 38) in the case of *Xenopus laevis*. To explain this, the mechanism of axis elongation in the tail-bud should be envisaged, and neural tube extension is one of the most applicable events independent of convergent extension from blastula to neurula. In the chick embryo, neural tube extension occurred at the underlying mesenchymal-to-epithelial transition (Shimokita and Takahashi, 2011). This phenomenon has never been reported in amphibians and is still different from our finding, but it is possible to say that the event of neural tube extension is independent of the convergent extension event in vertebrate embryos. In zebrafish, morphants of $GABA_A$-R changes in the proliferation pattern in the CNS and inhibits positive transcriptional feedback loop of *gad* (Gonzalez-Nunez, 2015). It has been reported that the GABA signal is related to the proliferation of embryonic stem cells and neural crest stem cells in mouse embryos by regulating S phase of cell cycling (Andang *et al.*, 2008). In pancreatic beta and neural cells, GABA is reported as a negative regulators of apoptosis (Prud'homme *et al.*, 2014; Zhang *et al.*, 2007). Using these mechanisms, GABA may

contribute to neural tube extension. Interestingly, the expression of *gad1* was not observed in the anterior dorsal midline region, which gives rise to future peripheral neurons (Fig. 1 B-G). In adults, GABA is expressed in peripheral neurons in addition to the brain and works as a neurotransmitter (Jessen *et al.*, 1979), indicating that the expression of GABA in early embryogenesis should have different roles from the neurotransmitter in adults. Our preliminary data shows that expression of GABA is also detected, but in a very small amount in the gastrula stage, and *gabr* is also expressed in the gastrula stage (Kaeser *et al.*, 2011). However, no phenotypic effect was observed in embryos treated with PTZ from stages 3 13 and from 13120 (Fig. 4 A-C), meaning that GABA should not be required for convergent extension, which is the event at the gastrula and neurula. Taken together, we conclude that axis elongation in the tail-bud may occur independently of convergent extension but is also regulated by GABA. What remains to be seen for future work is to clarify the molecular mechanism involved in axis elongation in the tail-bud caused by the GABA signal.

Strangely, we could not detect any effect on early embryos by the GABA treatment (Fig. 2 A,B; Supplementary Fig. S1) even though its inhibitors such as PTZ and PTX were active (Figs. 2-4; Supplementary Fig. S2), and GABA could rescue the effect by PTZ (Fig. 2). The reason why the GABA treatment had no activity in early embryogenesis might be because the speed of axis elongation in tail-bud embryos is already accelerated to a maximum velocity. In the case of convergent extension, some reports have suggested that elongation speed reaches a maximum. Both gain- and loss-of-function experiments for frizzled 7, which is considered to be a key regulator of convergent extension, lead to the same phenotypic effects such as inhibition of elongation in *Xenopus* (Djiane *et al.*, 2000). Both loss- and gain-of Lrp6, which is a Wnt co-receptor, also inhibits convergent extension in *Xenopus* embryos and explants (Tahinci *et al.*, 2007). Moreover, Dishevelled, which is a cytoplasmic phosphoprotein that acts directly downstream of frizzled receptors, is found to inhibit convergent extension in both gain- and loss-of-function studies (Wallingford *et al.*, 2002). In zebrafish, the activity gradient of bone morphogenetic proteins (BMPs) regulates convergent extension during gastrulation, but the difference in embryo length and mesendoderm length is not detected in both ventralized *chordino* and dorsalized *somitabun* mutants (Myers *et al.*, 2002). The same as for the case of convergent extension, the speed of axis elongation in the tail-bud may not be accelerated by any regulators. It is no wonder that intact speed cannot be increased because the elongation should be cooperative and followed with various other events and contributes to build a functional and well-organized stereostructure inside the embryo.

The metabolome screening performed in this study (Fig. 1 H,I) enabled us to find various other small ligand molecules in embryonic stages in addition to L-glutamate and GABA (data not shown). It is very hard to believe that none of these small ligand molecules have a role for early embryogenesis. An experiment on the role of these other small ligand molecules might contribute answers to missing pieces in the puzzle of developmental biology in the near future.

Materials and Methods

Embryology and histology

Xenopus laevis embryos were obtained by *in vitro* fertilization. The details of *in vitro* fertilization, as well as the protocol for histological sectioning, are described in Ohata *et al.*, 2014.

Chemical treatments of embryos

49.74 mg of PTZ powder (#P6500, Sigma-Aldrich, USA) was dissolved in 1 ml of 0.1x SS (360 mM). 60.2 mg of PTX powder (#C0375, Tokyo Chemical Industry, Japan) was dissolved in 1 ml of DMSO (dimethyl sulfoxide) (100 mM). 103.12 mg of GABA powder (#G0048, LKT Labs, USA) was dissolved in 1 ml of 0.1x SS (1 M). 89.09 mg of alanine powder (#010-01042, Wako, Japan) was dissolved in 1 ml of 0.1x SS (1 M). 75.06 mg of glycine powder (#077-00735, Wako) was dissolved in 1 ml of 0.1x SS (1 M). 180.16 mg of D-glucose powder (#047-00592, Wako) was dissolved in 1 ml of 0.1x SS (1 M). Each solution was diluted with 0.1x SS into the proper concentration. For culturing of embryos and explants, we used special coated plates to avoid binding of embryos and explants on the plastic plate. 12% poly 2-hydroxyethyl methacrylate (Poly-HEMA) solution (#18894, Polysciences, USA) was diluted to 4% with ethanol. 500 μl of 4% of Poly-HEMA solution was spread on a 40-mm diameter plastic plate, immediately removed, and then the plate was dried out. The chemical treatments were performed in the Poly-HEMA coated plates with 5 ml of solution including reagents. Embryos were exposed to each solution from stage 8 to the stages designed for observation unless otherwise specified in the text or figure legends.

Whole mount in situ hybridization

To make an RNA probe, the DNA fragment coding partial sequence of *gad1* was cloned by PCR using the following primers. gad1-fw: 5'-GGAGAAGATTGTAGTAGGGAGCTAAACTATCCATTTG-3'. gad1-rv: 5'-GCGAGGATGTCCTGTTCTGACTCCATACTTG-3'. This DNA fragment was subcloned into pTAC2 plasmid, and 10 μg of pTAC2-gad1 was digested with XhoI and transcribed by SP6 RNA polymerase (#10810274001, Roche, Switzerland). The embryos were fixed with MEMFA (100 mM MOPS, pH 7.4, 2 mM EDTA, 1 mM $MgSO_4$, 3.7% v/v formaldehyde) for 1 h after removal of the fertilization envelope. The protocol for whole mount *in situ* hybridization is described in http://kerolab.jp/protocol.html.

Metabolome analysis

For the sake of convenience, we called the dorsal part of the tail-bud embryo, which lost most of the yolk-rich region and lateral-to-ventral mesoderm, as the dorsal tail-bud explant (DTE, Fig. 1H). 10 DTEs were isolated, put into 300 μl of 0.1x SS in a 1.5 ml siliconized tube, and cultured for 8 h at 22 °C. 250 μl of supernatant was centrifuged at 20,000 g for 15 min, and the final 200 μl of supernatant (sample solution) were stored for metabolome analysis.

A total of 400 μl methanol with three internal standards (IS), 25 μM L-methionine sulfone (#502-76641, Wako), 25 μM CSA (D-Camphol-10-sulfonic acid) (#037-01032, Wako), and 25 μM MES (2-morpholinoethanesulfonic acid, monohydrate) (#349-01623, Dojindo, Japan) was added to 100 μl of culture medium and mixed very well, and then 500 μl of chloroform and 200 μl of mili-Q water were added. This mixture was centrifuged at 20,000 g at 4 °C for 15 min, and 400 μl of the aqueous phase were additionally centrifuged using a 5-kDa ultrafiltration tube (UFC3LCCNB, HMT, Japan) at 9,100 g at 20 °C for 2.5 h. This filtrated solution was dried out by mild centrifugation at 40 °C for 1.5 h, dissolved with water containing two IS, 200 μM 3-aminopyrrolidine (#404624, Sigma-Aldrich) and 200 μM of trimesate (#206-03641, Wako), and then applied to capillary electrophoresis-time of flight mass spectrometry (CE-TOF-MS). The protocol for CE-TOF-MS is described in Soga *et al.*, 2006, 2009. For quantification, the internal standard method was used. Specifically, metabolite standards (STD) and internal standards (IS), were measured at first, and then the samples were analyzed. The concentration of each metabolite was calculated using the following equation:

Metabolite concentration in suspension (μM)=
(Area of sample) / (ISArea of sample) ÷ (Area of STD)/(ISArea of STD) × STD conc. × (IS conc. in suspension) / (IS conc. in STD) × MeOH vol.+ suspension vol. / suspension vol.

Abbreviations: conc, concentration; vol, volume.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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