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ORIGINAL ARTICLE

CRH-R1 and CRH-R2 differentially modulate dendritic outgrowth of hippocampal neurons

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Abstract Corticotropin-releasing hormone (CRH) has been implicated to be involved in the development of dendrites in brain. In the present study, we examined the effect of CRH on dendrite outgrowth in primary cultured hippocampal neurons and defined the specific CRH receptor subtype involved. Treatment of neurons with increasing concentration of CRH resulted in an increase in the total dendritic branch length (TDBL) of neurons compared with untreated neurons over 2–4 days period of treatment. These effects can be reversed by the specific CRH-R1 antagonist antalarmin but not by the CRH-R2 antagonist astressin 2B. Treatment of neurons with urocortin II, the exclusive CRH-R2 agonist, significantly decreased TDBL of the cultured neurons. These effects can be reversed by the CRH-R2 antagonist astressin 2B. Our results suggest that CRH-R1 and CRH-R2 differentially modulate the dendritic growth of hippocampal neurons in culture.

Keywords Corticotropin-releasing hormone · Corticotropin-releasing hormone receptors · Dendrite · Hippocampus

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Introduction

During nervous system development, formation of a network of neuronal circuitries has profound functional implications. At the level of a single neuron, the structural organization of the postsynaptic surface, the dendritic arbor, is one of the critical determinants of neuronal circuitry besides the axon projection of presynaptic neurons [1]. The dendritic patterns of a neuron determine the number and arrangement of its synaptic inputs and the way a neuron integrates and processes its inputs [2, 3]. Thus, it is important to understand the mechanisms controlling the dendritic branch number, distribution, and length.

The dendritic arbor development is controlled by the intrinsic genetic programs as well as extracellular signals [4, 5]. Intrinsic factors determine the extent and pattern of dendritic outgrowth [6, 7]. However, extracellular factors including neurotransmitters, neurotrophins, and hormones also regulate extent and rate of dendritic extension, retraction, or stabilization [8–10]. For instance, glucocorticoids can induce retraction of apical dendrites of hippocampal neurons [11, 12].

Corticotropin-releasing hormone (CRH), a 41-amino-acid polypeptide, is a critical neurotransmitter and/or neuromodulator in the brain, which regulates a wide spectrum of autonomic, electrophysiological, and behavioral responses to stress [13]. CRH has also been implicated to regulate synaptic plasticity including long-term potentiation and dendritic development [14]. There may be conflict regarding the effect of CRH on dendrites development. Cibelli et al. [15] have shown that CRH stimulates neurite outgrowth of a catecholaminergic immortalized neuron in culture. In contrast, Chen et al. [16] reported that CRH suppresses the dendrites growth in hippocampal organotypic cultures.

Corticotropin-releasing hormone exerts its actions via two G-protein coupled receptors, CRH receptor type 1 (CRH-R1) and type 2 (CRH-R2). More recently, Chen et al. [16, 17] reported that CRH-R1 knockout results in abnormal shape of dendritic trees in hippocampus, suggesting that CRH-R1 is critical for dendritic development. So far, the role of CRH-R2 in the dendritic development remains unknown. Thus, we conducted the experiments to explore the effect of CRH on dendrite outgrowth in cultured hippocampal neurons and elucidate the role of CRH-R1 and CRH-R2 in the regulation of dendrite development.

Materials and methods

Chemicals

Corticotropin-releasing hormone, antalarmin, and astressin 2B were purchased from Sigma-Aldrich (St Louis, MO). Urocortin II was obtained from Bachem California Inc. (Torrance, CA). DMEM, B27, neurobasal medium, fetal bovine serum (FBS), horse serum, and trypsin were supplied by Life Technologies (Grand Island, NY). Antibody against microtubule-associated protein 2 (MAP2) was purchased from Neomarkers Biotechnology (Neomarkers, Fremont, CA). Antibody against NMDA receptor subunit 2A (NR2A) was from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of hippocampal neuron cultures

All animal procedures were approved by the Ethical Committee of Experimental Animals of Second Military Medical University, China. Procedures were designed to minimize the number of animals used and their suffering. Primary hippocampal neurons were cultured as described previously [18]. Briefly, the hippocampi were dissected from neonatal (P1) Sprague-Dawley rats in ice-cold dissection solution containing sucrose/glucose/HEPES (136 mM NaCl, 5.4 mM KCl, 0.2 mM Na_2HPO_4, 2 mM KH_2PO_4, 16.7 mM glucose, 20.8 mM sucrose, 0.0012% phenol red, and 10 mM HEPES, pH 7.4), and then incubated with 0.125% trypsin at 37°C for 20 min. Single cell suspension was obtained by mechanically dissociation using a Pasteur pipette with a fire-narrowed tip in DMEM containing 10% heat-inactivated FBS and 10% horse serum. Cells were then plated at a density of 1×10^5 cells/cm^2 on poly-L-lysine coated 6-well plates or glass coverslips. Culture was maintained in 5% CO_2 at 37°C in DMEM containing 10% heat-inactivated FBS and 10% horse serum overnight, and then the culture media were changed to serum-free B27/neurobasal medium. Half of the medium was replaced with fresh medium every 3 days. Neurons were cultured 7 days before use in experiments.

Transient transfection

Transient transfections were performed using SofastTM (Sunma Biotech, Xiamen, China) cationic polymer transfection reagent according to the manufacturer's protocols. Enhanced green fluorescent protein-N3 (EGFP-N3) expression vector was purchased from Clontech (Mountain View, CA). At 7 days in vitro (7DIV), the hippocampal neurons were transfected with EGFP-N3 plasmid DNA (0.4 μg DNA/well). Three hours later, culture media were changed to fresh media. After 24 h, the neurons were treated with CRH, antalarmin, astressin 2B, or urocortin II for 2 or 4 days, and then the length of dendrite was analyzed.

Immunofluorescence analysis

The cells were fixed in 4% paraformaldehyde for 1 h after washing with PBS. Fixed cells were washed with PBS and incubated with 10% BSA for 1 h. Then cells were incubated with a mouse monoclonal antibody against MAP2 (Neomarkers, ms-249-p0) or a rabbit antibody against NR2A (Santa Cruz, sc-9056) at a dilution of 1:500 overnight at 4°C. All dilutions were made in PBS containing 1% BSA. Subsequently, the specimens were washed with PBS three times and then incubated with rhodamine-conjugated anti-mouse or rhodamine-conjugated anti-rabbit IgG (1:100) at 37°C for 1 h in the dark. For negative controls, the primary antibody was substituted with a normal IgG in same dilution. Results were viewed under fluorescent microscope using appropriate filters. To determine the number of cells expressing MAP2 and NR2A in cultured hippocampal neurons, a threshold of average cytoplasmic density level of immunoreactive product was set and determined using an image of negative control neurons (normal IgG control). The areas of all measured cells were obtained at the same time, and for each cell, the optical density of immunoreactive product and the cell areas were plotted.

Microscope

The low efficacy of transfection (~0.1%) allowed single-labeled neuron to be observed under a fluorescence microscope. Neurons expressing EGFP were imaged at ×200 using an Olympus Optical IX70 microscope (Tokyo, Japan) with a Roper Scientific CCD cooled camera and RS Image software. The total dendritic branch length (TDBL) was analyzed with Image J software (http://rsb.info.nih.gov/ij/) manually, and data from axons were not included. Dendrites were distinguished from axons by the following criteria [19]: (i) dendrites emit branches at an angle <45°, while axons typically bifurcate at an angle close to 90°; (ii) the dendrites are usually shorter than axons, and axons are usually finer and much longer; and (iii) dendrites are

positive for MAP2 immunostaining. Only neurons with the defined dendrites were used for further analysis. At the end of each experiment, the dendrites measured were verified by immunostaining with MAP2. The dendrites initiating from the cell soma were defined as primary dendrites [20]. In each culture, we picked up the cells that were clearly staining for EGFP calculating TDBL. A total of 120 neurons from three independent cultures were analyzed in each treatment. In addition, the neurons with MAP2 staining were also used to measure the TDBL. We randomly picked up 30−40 neurons clearly staining for MAP2 for calculation of TDBL in each culture. A total of 120 neurons from three independent cultures were analyzed in each treatment.

Statistically analysis

All data were expressed as mean ± SEM. One-way ANOVA plus Dunnett's test was used to compare the mean values of TDBL. $P < 0.05$ was considered significant.

Results

CRH increases dendritic outgrowth in cultured hippocampal neurons

Immunofluorescence analysis showed that a lot of neurons were MAP2 positive. More than 100 neurons were calculated. There were about 91% (153 positive cells/168 total cells) were MAP2 positive. In addition, the staining for NR2A, a subunit of *N*-methyl-D-aspartate (NMDA) receptors, was examined to define the cell type. It was found that 84% (147 positive cells/174 total cells) of the cells were NR2A positive (Fig. 1).

As shown in Fig. 2, neurons expressing EGFP displayed clearly the branch and the length of dendrites. Cultured neurons were treated with increasing concentration of CRH (1 pmol/l to 10 nmol/l) for 2 and 4 days. The TDBL of 120 EGFP positive neurons were evaluated. It was found that CRH treatment resulted in a significant increase in TDBL over a 2- or 4-day treatment period for all dosages of CRH (2 day: $F_{(3,476)} = 40.3$; $P < 0.01$; 4 day: $F_{(3,476)} = 43.4$; $P < 0.01$). CRH (10 nmol/l) treatment for 2 days significantly increased TDBL about 84% compared with untreated cells (674 ± 25 μm for CRH, 366 ± 16 μm for control, $n = 120$, Fig. 3a). CRH (10 nmol/l) treatment for 4 days significantly increased TDBL about 75% compared with untreated cells (893 ± 34 μm for CRH, 511 ± 21 μm for control, $n = 120$, Fig. 3a). There was no significant difference in number of primary dendrites between neurons treated with CRH for 2 days and the control neurons ($F_{(3,\ 216)} = 0.42$; $P > 0.05$). The results showed that the number of primary dendrites was 4.4 ± 0.16 in the absence of CRH and 4.5 ± 0.19 in the presence of CRH (10 nmol/l), respectively ($n = 55$).

In order to further confirm the effects of CRH on dendritic growth, the TDBL was also analyzed in neurons stained with MAP2. Treatment of neurons with increasing concentration of CRH for 2 days, cells was then fixed and

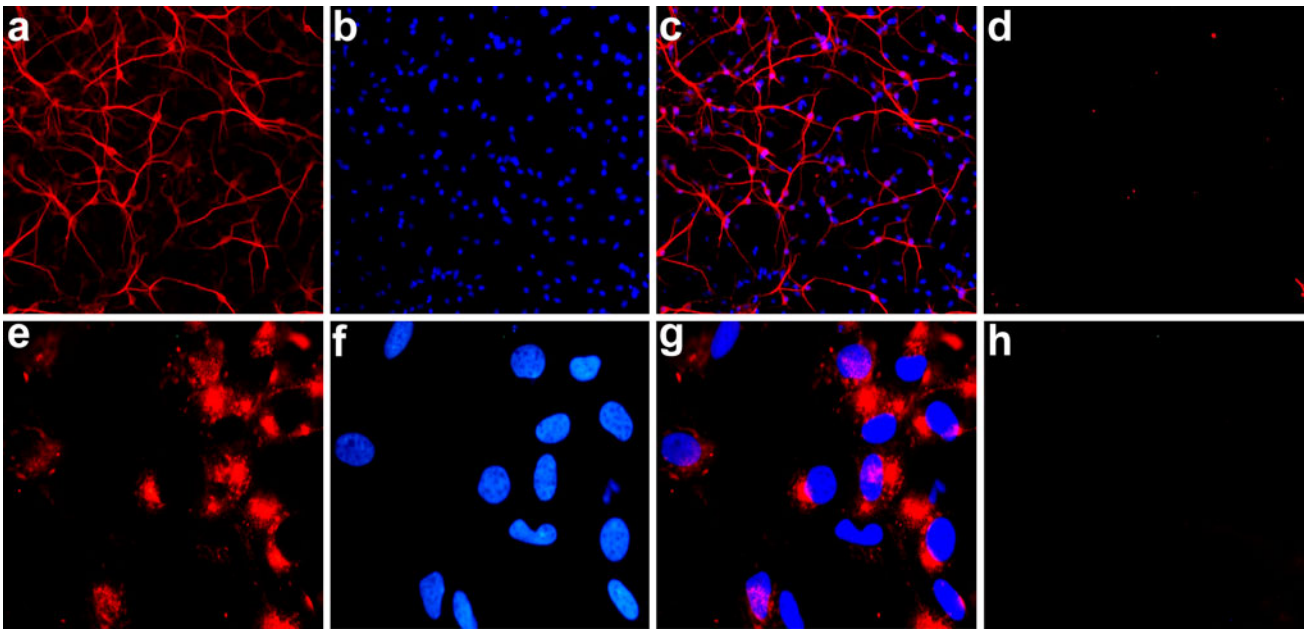

Fig. 1 Expression of MAP2 and NR2A in cultured hippocampal cells. The cultured cells were stained with specific antibodies against MAP2 (**a**–**d**) and NR2A (**e**–**h**) as described in "Materials and methods" section. **a** positive staining of MAP2, **e** positive staining of NR2A, **b** and **f** the cells immunostained for hochest, **c** MAP2 and hochest overlay, **g** NR2A and hochest overlay, **d** and **h** negative controls: primary antibody was substituted with normal IgG. Original magnifications, ×100 (**a**–**d**) or ×400 (**e**–**h**)

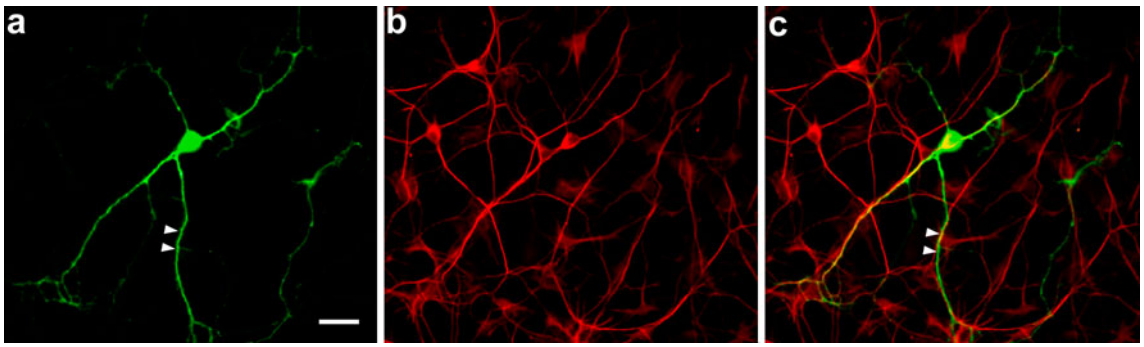

Fig. 2 Identification of dendrites in cultured hippocampal neurons. The cultured hippocampal neurons were transfected with EGFP plasmid DNA at 7DIV. **a** cultured hippocampal neurons expressing EGFP at 10DIV, **b** the same cells immunostained for MAP2, **c** EGFP and MAP2 overlay. *Arrowheads* indicate the location of axon. *Scale bars* 20 μm (magnification, ×200)

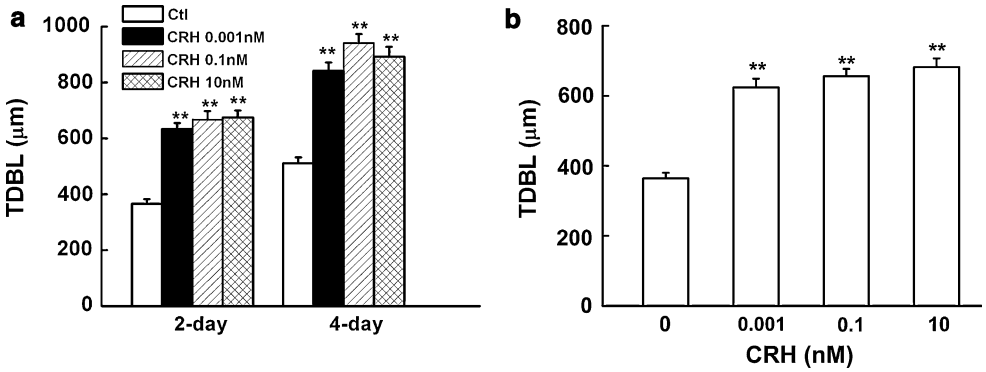

Fig. 3 Effects of CRH on TDBL in cultured hippocampal neurons. Neurons were treated with increasing concentrations of CRH for 2 and 4 days. Data of TDBL were obtained from cells transfected with EGFP plasmid (**a**) and from cells stained with MAP2 (**b**). Data were presented as means ± SEM ($n = 120$). $^{**}P < 0.01$ versus control. *Ctl* control

stained with MAP2 antibody. It was found that CRH treatment significantly increased the TDBL for all dosages of CRH ($P < 0.01$ vs. control, $n = 120$; Fig. 3b). CRH treatment did not affect the number of primary dendrites. The number of primary dendrites was 4.5 ± 0.16 in the absence of CRH and 4.3 ± 0.16 in the presence of CRH (10 nmol/l), respectively ($n = 55$).

The effect of CRH is blocked by CRH-R1 antagonist

Our previous study had shown that cultured hippocampal neurons express both CRH-R1 and CRH-R2 [21]. To determine the role of subtype of CRH receptor, the specific antagonist of CRH-R1 [22] or CRH-R2 [23] was used. As shown in Fig. 4, the CRH-R1 antagonist antalarmin (100 nmol/l) completely blocked the effect of CRH (10 nmol/l). The TDBL of EGFP- and MAP-positive neurons treated with CRH-R2 antagonist astressin 2B (100 nmol/l) plus CRH (10 nmol/l) was not significantly different from control ($P > 0.05$ vs. control).

CRH receptor agonist urocortin II inhibits dendritic outgrowth

To further clarify whether CRH-R2 is involved in the effect of CRH on dendritic growth, we examined the effect of the

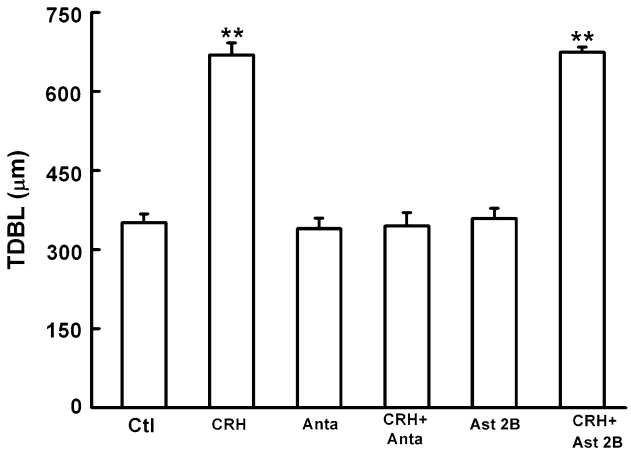

Fig. 4 Effects of CRH receptor antagonists on CRH-induced increase in TDBL. Neurons were treated with CRH (10 nmol/l), antalarmin (100 nmol/l), and CRH (10 nmol/l) in combination with antalarmin (100 nmol/l), astressin 2B (100 nmol/l), or CRH (10 nmol/l) in combination with astressin 2B (100 nmol/l) for 2 days ($n = 120$). Data are normalized to the control and presented as means ± SEM. $^{**}P < 0.01$ versus control. *Ctl* control; *Antalamin* Anta, *astressin 2B* Ast 2B

CRH-R2 exclusive agonist urocortin II on TDBL of cultured cells. As shown in Fig. 5, application of urocortin II (10 fmol/l to 100 pmol/l) caused a dose-dependent decrease in TDBL over a 2 day treatment period, maximal inhibition effect of urocortin II was obtained at a

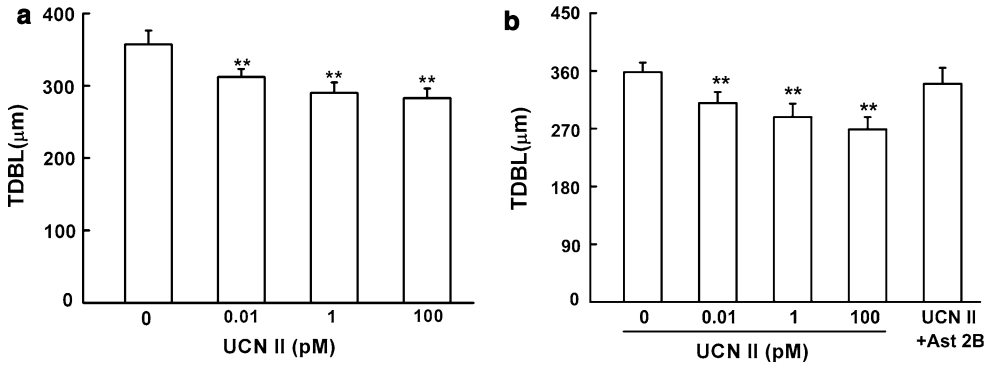

Fig. 5 Effects of urocortin II on TDBL in cultured hippocampal neurons. Neurons were treated with increasing concentrations of urocortin II for 2 days. Data of TDBL are obtained from cells transfected with EGFP plasmid (**a**) and from cells stained with MAP2 (**b**). Data were presented as means ± SEM ($n = 110$). $^{**}P < 0.01$ versus control. *Urocortin II* UCN II, *astressin 2B* Ast 2B

concentration of 100 pmol/l, which resulted in 25% decrease in TDBL compared with control cells ($P < 0.01$ vs. control). This effect was blocked by CRHR2 antagonist astressin 2B (Fig. 5b). There was no significant difference in number of primary dendrites between neurons treated with urocortin II and the control neurons ($P > 0.05$ vs. control, for all comparisons). The number of primary dendrites was 4.5 ± 0.19 in the absence of urocortin II and 4.6 ± 0.16 in the presence of urocortin II (10 nmol/l), respectively ($n = 55$).

Discussion

We have demonstrated that more than 80% of the cultured cells obtained from hippocampus of newborn rats were NR2A positive staining. It has been shown that NR2A could be a marker of CA3 pyramidal cells [24]. Thus, most of cultured cells might be pyramidal cells although we could not exclude other neuron contamination such as inhibitory interneuron.

Treatment of neurons with CRH significantly increased TDBL but not affect the number of primary dendrites, suggesting that CRH mainly modulate the elongation of dendritic processes. The stimulatory effect of CRH on TDBL was completely reversed by the CRH-R1 antagonist. However, the CRH-R2 antagonist did not block CRH-increased TDBL. Urocortin II, the exclusive CRH-R2 agonist, decreased the TDBL. The effects can be reversed by the CRH-R2 antagonist. These findings suggest that CRH-R1 and CRH-R2 differentially modulate dendritic growth of hippocampal neurons in culture.

Craig and Banker [25] proposed that hippocampal neurons of rat proceed through five defined stages of growth and polarization following plating in vitro. At stage 1, the neurons display one or more lamellipodia on their surface shortly after plating. At stage 2, neurons extend short undifferentiated neurites. At stage 3, one of the initially equivalent neurites grows rapidly and becomes the axon. A few days after the axon has begun its rapid growth, the remaining neurites elongate and develop into dendrites during stage 4. About 7 days after plating, stage 5 is marked by the formation of a neuronal network and synaptogenesis. Thus, we chose cultured hippocampal neurons at stage 5 to do transfection. It was shown that the neurons which expressed EGFP clearly displayed the dendrites and axons.

Both CRH-R1 and CRH-R2 are identified in hippocampal neurons, in particular, reside mainly on dendritic spines of pyramidal cells [26]. Our previous study also found that more than 90% cultured hippocampal cells express CRH-R1 and CRH-R2, and they are also identified in dendrites of cultured neurons [21]. In the present study, we demonstrated that CRH-R1 mediated stimulatory effect of CRH on dendritic growth of hippocampal neurons in culture. Given that most cells are pyramidal cells in our culture system, our data suggest that CRH may act CRH-R1 to stimulate the dendritic outgrowth of pyramidal cells in culture although we could not exclude the effect of CRH on other cell types. However, Chen et al. [16] have demonstrated that CRH-R1 deficient mice show exuberant dendritic trees in hippocampal principal cells, and CRH reduces while CRH-R1 antagonist enhances the dendritic length in hippocampal organotypic slice cultures. The differences in experimental conditions might be responsible for this discrepancy, in particular, the culture system difference ("primary cell culture in our study" vs. "organotypic slice culture in Chen et al. study"). In primary hippocampal cell culture, the cells were dispersed from hippocampal tissue. Thus, the microenvironments of hippocampal neurons in primary culture are different from organictypic slice culture, which might be responsible for the discrepancy of CRH effect in these two culture systems. In addition, the study of Swinny et al. has demonstrated

that intermittent exposure of CRH and urocortin induces more dendrite outgrowth of Purkinje cells in organotypic cerebellar slice culture. Conversely, constant exposure to CRH and urocortin inhibits dendritic outgrowth [27]. It may suggest that the effects of CRH and its related peptides be dependent on the type of neurons.

Corticotropin-releasing hormone receptors are also activated by urocortin family of peptides, with urocortin I having equal affinity for both CRH-R1 and CRH-R2, and urocortin II and urocortin III having exclusive affinity for the CRH-R2 [28]. It was found that CRH-R1 activation exerts the inhibitory effect on the activity of *N*-methyl-D-aspartate (NMDA) in hippocampal neurons [21]. CRH-R2 activation potentiates the activity of NMDARs in dopamine neuron in the ventral tegmental area [29]. Our previous study has demonstrated that the cultured hippocampal neurons express both CRH-R1 and CRH-R2 [21]. The present study showed that urocortin II inhibited dendritic growth, and this effect was blocked by CRH-R2 antagonist. Thus, CRH-R1 and CRH-R2 in hippocampal neurons mediate opposite effect on dendrite growth. At current stage, the mechanisms responsible for differential regulation of dendrite growth by CRH-R1 and CRH-R2 are not known. It has been demonstrated that CRH-R could couple to multiple G proteins including Gs, Gi, and Gq/11 and then go on to induce multiple signaling pathways [30]. The signaling pathways responsible for the effects of CRH-R1 and CRH-R2 on dendrite growth should be investigated in following studies. In addition, it was found that CRH had much stronger stimulating effect on dendritic outgrowth by doubling the TDBL than urocortin II which only reduced dendritic elongation by about 20%. It is of interesting to explore the discrepancy in such effects of CRH and urocortin II in the future.

CRH-R1 and CRH-R2 have been implicated in modulation of learning and memory in responses to stress [30, 31]. In addition, studies by Chen et al. indicate that CRH is involved in memory defects and hippocampal dendritic spine loss after acute stress [16, 17, 32]. Our findings that CRH-R1 and CRH-R2 activation have differential actions on dendritic growth in hippocampal neurons suggest that CRH and its related peptides be involved in modulation of hippocampal synaptic connectivity. Nevertheless, the effects of CRH-R1 and CRH-R2 on dendritic development in vivo need to be done in the CRH-R1 and CRH-R2 deficient mice.

In conclusion, CRH stimulates dendritic outgrowth of cultured hippocampal neurons via CRH-R1. In contrast, CRH-R2 activation results in an inhibition in dendritic growth. Our results suggest that CRH-R1 and CRH-R2 differentially modulate the dendritic growth of hippocampal neurons in culture.

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Conflict of interest None.

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