

Effects of Hochu-ekki-to (Bu-Zhong-Yi-Qi-Tang), a Kampo medicine, on serotonin 2C subtype receptor-evoked current response and the receptor mRNA expression

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Abstract

Hochu-ekki-to (HET, Bu-Zhong-Yi-Qi-Tang, Formula repletionis animelis et suppletionis medii) is one of the Kampo medicine which has been used for improving disturbance and imbalance in the homeostatic condition of the body. Repeated treatment with HET (5.7 g/kg as estimated galenicals, p.o.) enhanced the serotonin 2C subtype receptor (5-HT₂CR) mRNA expression in rat brain as effectively as 20 mg/kg imipramine p.o. HET at only 5 μ g/ml as estimated galenicals evoked two types of inward current: one was of the metabotropic type and the other was of the ionotropic type. The metabotropic type current, which was expected to have strong intensity compared with that evoked by serotonin, was blocked by prior treatment with 10 μ M mianserin, an antagonist of 5-HT₂CR. These results suggest that HET contains strong 5-HT₂CR agonistic factor(s).

Key words Antidepressive effects, 5-HT₂C receptor, gene expression, current response, in situ hybridization, *Xenopus* oocyte, repeated administration.

Abbreviations HET, Hochu-ekki-to (Bu-Zhong-Yi-Qi-Tang), 補中益気湯; 5-HT₂CR, serotonin 2C subtype receptor; MBS, modified Barth's solution; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; SSC, standard saline citrate.

Introduction

Hochu-ekki-to (HET, Bu-Zhong-Yi-Qi-Tang, Formula repletionis animelis et suppletionis medii) is one of the most often used Japanese-Chinese traditional herbal medicines (Wakan-yaku), which was first produced by Dong Yuan Li about 750 years ago. Recent studies have shown that HET has effects of immune system activation,¹⁻³⁾ anti-tumor activity⁴⁻⁶⁾ and improvement of men's infertility through activation of sperm motility.⁷⁾ We previously reported that HET prolonged the immobilization time in a forced swimming test, suggesting that HET has an antidepressive effect.⁸⁾

We have also reported about the effects of antidepressants on 5-HT₂C subtype receptor (5-HT₂CR) signal transduction and gene expression. Antidepressants which are thought to act on the serotonergic system inhibited the chloride current induced by 5-HT₂CR stimulation in *Xenopus* oocytes injected with mRNA⁹⁾ and inhibited Cl efflux from the oocytes.¹⁰⁾ The generation of inositol phosphates in 5-HT₂CR cDNA-transfected COS-7 cells was also inhibited by antidepressants.¹¹⁾ These results suggest that antidepressants which act on the serotonergic system are antagonists of 5-HT₂CR, as also described by other groups.¹²⁾ The necessity of long-lasting and repeated treatment with antidepressants for several weeks to determine their therapeutic effort suggests that some

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physiological mechanisms which are changed during long-lasting exposure to antidepressants are involved in. Therefore, we examined the effects of chronic treatment with antidepressants on gene expression and found that repeated oral administration of antidepressants possessing 5-HT₂CR-blocking activity elevated the level of 5-HT₂CR mRNA in rat brain.^{13,14)}

In this report, we studied the effects of HET on 5-HT₂CR mRNA expression in rat brain as a long-term effect and on 5-HT₂CR stimulation-induced Cl⁻ current in mRNA-injected *Xenopus* oocytes as an acute effect. Since HET has been used clinically for more than 750 years in China and Japan, and recently the Food and Drug Association (FDA) of the USA announced that traditional herbal drugs, also called alternative medicines, are thought to be drugs analogous to pure chemical compounds, we tried to find the pharmacological basis of HET activity and found effects on 5-HT₂CR-evoked current response and gene expression.

Materials and Methods

Extraction of HET: HET contains the following 10 galenicals. All galenicals were purchased from Tochimoto Tenkai-do (Osaka, Japan). The daily dose of each galenical, the location where its plant source was grown and the lot number are shown; 4 g Ginseng Radix (Korea: 300190), 4 g *Atractylodes Rhizoma* (Zhejiang Sheng, China: 220295), 3 g *Astragali Radix* (Shan Xi Sheng, China: 100395), 3 g *Angelicae Radix* (Yamato, Japan: 120695), 2 g *Citri Leiocarpae Exocarpium* (Shikoku, Japan: 220894), 2 g *Zizyphi Fructus* (He Nan Zheng, China: 300395), 2 g *Zingiberis Rhizoma* (Yun Nan Sheng, China: 290788QC), 2 g *Bupleuri Radix* (Nara, Japan: 220290), 1.5 g *Glycyrrhiza Radix* (Xi Bei, China: 071194) and 1 g *Cimicifugae Rhizoma* (Ji Lin Sheng, China: 100288QC). Each galenical used in this study is kept in the Analytical Research Center for Ethnomedicines, Institute for Natural Medicine, Toyama Medical and Pharmaceutical University, Japan. A total of 98 g of these galenicals, which was estimated to be needed for 4 days of administration was used for extraction with 900 ml of boiling water for 90 min. Galenicals were

removed while the solution was still hot, and the solution was frozen, followed by freeze drying. The yield of the extraction was 34 %. The dried extract of HET was dissolved in distilled water and orally administered to the rats. HET extract was dissolved in the test buffer (see below) in the case of the electrophysiological study. The dose (mg/kg) or concentration (mg/ml) of HET is shown by estimated galenical weight calculated based on the % yield.

Production of the cRNA probe against 5-HT₂CR:

To obtain the digoxigenin-labeled RNA probe corresponding to the sequence of the third intracellular loop domain, which is unique to each G protein-coupled receptor, the pBluescript II KS(-) vector containing rat 5-HT₂CR cDNA was rearranged using restriction enzymes, DNA polymerase and ligase as described previously.¹³⁾ The resultant 275 bp 5-HT₂CR cDNA fragment (nucleotides 1355-1630) was inserted between nucleotides 657 and 753 of the pBluescript vector. The reconstituted vector, which was used to make a probe, was cut by Hinc II at nucleotide 1368 of the 5-HT₂CR cDNA for the T3 RNA polymerase reaction to make antisense RNA or by Kpn I at nucleotide 759 of the vector for the T7 RNA polymerase reaction to make sense RNA. The reactions to make these probes were performed essentially as described previously.¹³⁾

In situ hybridization: Imipramine or vehicle (water) was administered orally at 15:00 for 4 days to male Wistar rats (8 weeks old, 220-250 g, Japan SLC Inc., Hamamatsu, Japan). One hour after the last administration, the rats were killed by decapitation. Their brains were removed, immediately frozen with powdered dry ice, and stored for 1 day at -80°C. Frozen brain sections (16 µm) were cut using a cryostat, mounted onto gelatin-coated slides, and air-dried. Before hybridization, sections were fixed with 4 % (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, followed by 0.5 µg/ml proteinase K treatment and acetylation. The sections were dehydrated through a graded ethanol series, placed in chloroform to remove fat, treated with 100 % ethanol and dried. After prehybridization, the sections were hybridized overnight with digoxigenin-labeled probe in a hybridization buffer consisting of 5×standard saline citrate (SSC: 150 mM NaCl, 17 mM sodium

citrate, pH 7.0), 50 % formamide, $2.7\times$ Denhardt's solution, 10 mM EDTA, 20 mM dithiothreitol, 0.25 mg/ml tRNA, and 10 % dextran sulfate at 55°C. The hybridized sections were washed with $2\times$ SSC at 55°C, treated with 50 μ g/ml RNase A for 30 min, washed with 50 % formamide/ $2\times$ SSC at room temperature, dehydrated in ethanol and dried. 5-HT₂CR mRNA hybridized with the digoxigenin-labeled probe was detected immunohistochemically using an alkaline phosphatase-conjugated antidigoxigenin antibody with 450 μ g/ml nitroblue tetrazolium and 175 μ g/ml X-phosphate as substrates. Gray scale images of stained sections were scanned and saved using a computer and Photoshop software. Quantitative analysis of the staining densities was performed using the NIH Image analysis system as described previously.¹³⁾

Oocyte injection and current recording: Defolliculated stage V-VI oocytes were prepared from *Xenopus laevis* (Hamamatsu Seibutsu, Shizuoka, Japan), as described previously.⁹⁾ Briefly, *Xenopus laevis* were anesthetized in ice-water and a lobe of the ovary was removed and placed in sterile modified Barth's solution (MBS: 88 mM NaCl, 1.0 mM KCl, 0.41 mM CaCl₂, 0.33 mM Ca (NO₃)₂, 0.82 mM MgSO₄,

2.4 mM NaHCO₃, 7.5 mM Tris-(hydroxymethyl) aminomethane, pH 7.6). Oocytes were then isolated and defolliculated with 1.5 mg/ml collagenase (Wako Pure Chemical Industries, Osaka) in Ca²⁺-free MBS at 22°C for 30 min. Total RNA was prepared from the whole brains of adult male rats (Japan SLC, Shizuoka, Japan), by the method of Chomczynski and Sacchi.¹⁵⁾ The mRNA was dissolved in sterile water at a final concentration of approximately 5 mg/ml and injected into the oocytes. The oocytes were incubated in MBS containing 2.5 units/ml penicillin and 2.5 μ g/ml streptomycin at 18°C for 2 days before recording currents. The MBS was replaced daily. The transmembrane currents were recorded using the two-electrode voltage-clamp method (GeneClamp 500, Axon Instruments, Foster City, CA). The voltage-monitoring electrode was filled with 3 M KCl and the current-passing electrode with 3 M ammonium acetate. An oocyte was positioned in a 50 μ l chamber and continuously perfused with MBS at 1.5 ml/min at room temperature (22-25°C). Healthy oocytes possessing negative membrane potentials exceeding -20 mV were used for the experiments, and the membrane potential was maintained at -60 mV. The drugs were applied

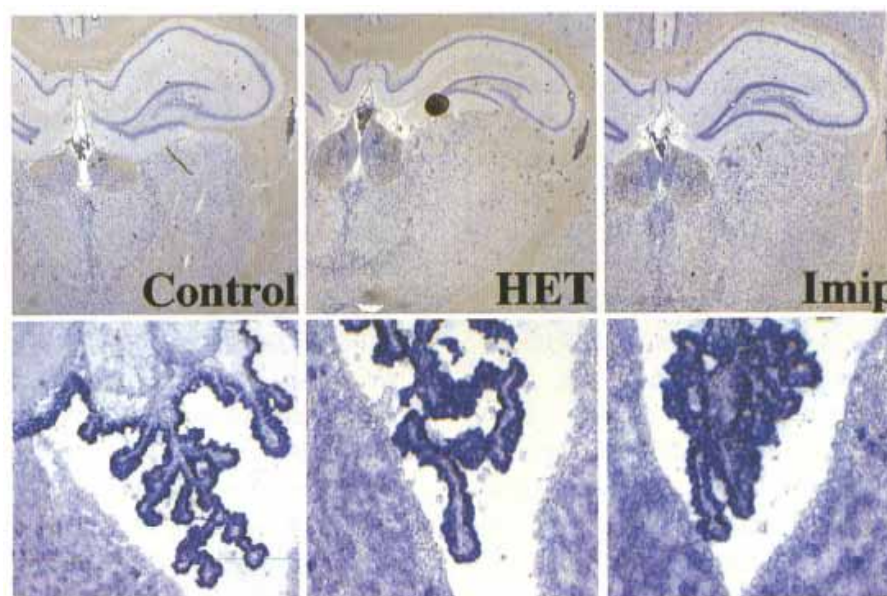


Fig. 1 Effects of repeated treatment with Hochu-ekki-to (HET) and imipramine (Imip) on 5-HT₂CR mRNA expression in the rat brain.

HET (5.7 g/kg as estimated galenicals, p.o.), imipramine (20 mg/kg p.o.) or water (control) was administered once daily for 4 days. Upper photos show slices at around bregma -2.30 mm. Lower photos show magnification of the choroid plexus in the third ventricle.

using ValveLink 8 (AutoMate Scientific Inc., CA, USA), which could immediately change the buffer solution to drug solution.

Results

Effects of repeated treatment with HET on 5-HT₂CR mRNA expression

In our previous study,¹³⁾ 4 days of treatment with 20 mg/kg imipramine p.o. enhanced the 5-HT₂CR mRNA expression in almost all parts of the rat brain where 5-HT₂CR was expressed. Therefore, we used imipramine as a positive control in the present experiments in which the effects of HET on 5-HT₂CR mRNA expression were examined. Four days of treatment with 5.7 g/kg HET p.o. stimulated the 5-HT₂CR mRNA expression as well as 20 mg imipramine p.o. (Fig. 1), although 0.57 g/kg HET p.o. had no effect. The enhancement was observed in almost all the parts of the brain expressing 5-HT₂CR: the choroid plexus of the third and lateral ventricles, the hippocampus, the habenular nucleus (Fig. 1), the amygdala, the piriform cortex (data not shown) and so on. Since the most abundant expression was observed in the choroid plexus, the quantitative analysis was performed in that area. The signal density was significantly enhanced by HET treatment, as well as by imipramine, to almost the same extent (Fig. 2).

*Effects of HET on 5-HT₂CR response in *Xenopus* oocytes injected with rat brain RNA*

Since antidepressants which are involved in the serotonergic system have a 5-HT₂CR antagonistic activity, we examined the effects of HET on the Cl current in *Xenopus* oocytes injected with rat brain RNA. 5-HT (1 μ M) elicited an inward current response and the response was blocked by prior treatment with 10 μ M mianserin, a typical 5-HT₂CR antagonist, or with 10 μ M imipramine for 3 min (Fig. 3), as previously reported.⁹⁾ Imipramine and mianserin caused no response by themselves. HET evoked the inward current even at only 5 μ g/ml (Fig. 3). The current showed two typical patterns: one was ionotropic type like the γ -aminobutylic acid (GABA),¹⁶⁾ glycine^{17,18)} and N-methyl-D-aspartate (NMDA)^{19,20)}-evoked current, which is evoked just after application of drug to oocyte and immediately

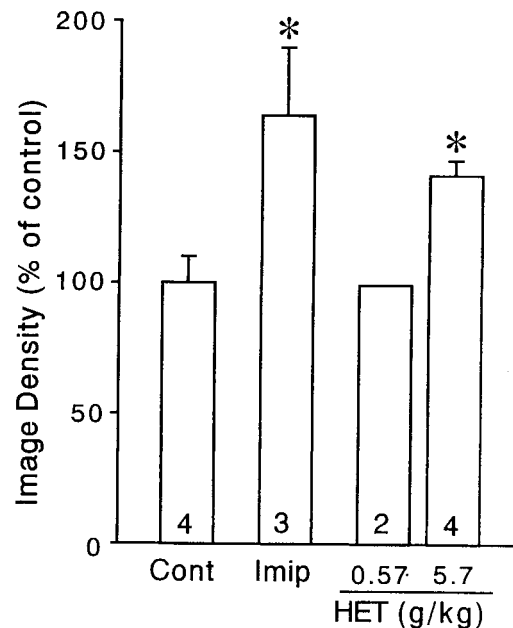


Fig. 2 Effects of repeated treatment with Hochu-ekki-to (HET) and imipramine (Imip) on 5-HT₂CR mRNA expression in the rat brain. HET (0.57 and 5.7 g/kg as estimated galenicals, p.o.), imipramine (20 mg/kg p.o.) or water (Cont) was administered once daily for 4 days. The staining densities were quantified using an NIH Image scanner. Values represent the mean \pm S.E.M., expressed as percent of the blank (the area not labeled by 5-HT₂C probe) of 2–4 experiments. An asterisk indicates $P < 0.05$ for comparison with control (water treated).

disappears after drug removal. The other one was metabotropic type, like the 5-HT and acetylcholine current, in which there is a delay before the current is evoked because of the time needed for metabotropic steps.^{21,22)} The metabotropic current induced by 5 μ g/ml HET was blocked by prior treatment with 10 μ M mianserin, although the ionotropic current was regenerated when HET was applied again after treatment with mianserin (Fig. 3).

Discussion

Repeated oral administration of HET, which is expected to have an antidepressive effect based on our previous report,⁸⁾ elevated the 5-HT₂CR mRNA expression in the rat brain as did other antidepressants with 5-HT₂CR blocking ability.^{13,14)} Since the 5-HT₂CR reconstitution system of *Xenopus* oocytes injected with rat brain mRNA was used to estimate

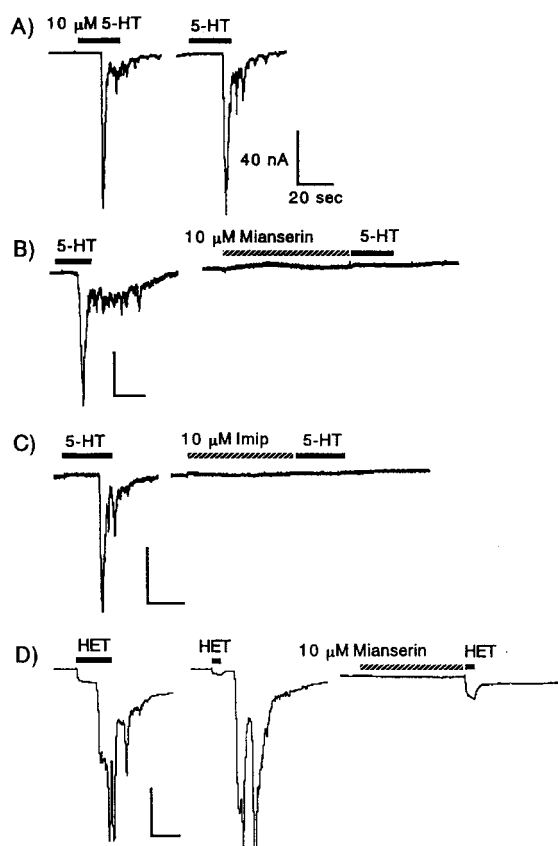


Fig. 3 Effects of HET on 5-HT-evoked current in *Xenopus* oocytes injected with rat brain RNA. Transmembrane currents were measured by the voltage clamp method, holding at -60 mV. Each drug was applied during the period shown by closed (agonist) or hatched (antagonist) bars. A) $10 \mu\text{M}$ 5-HT was applied twice with a 10 min interval. B,C) $10 \mu\text{M}$ mianserin or 10 mM imipramine treatment was performed about 1 min prior to the second application of 5-HT. D) $5 \mu\text{g/ml}$ HET was applied twice with a 10 min interval followed by a third application after treatment with $10 \mu\text{M}$ mianserin for about 1 min.

the 5-HT₂CR blocking activity of antidepressants in our previous experiments,^{9,10)} we also used this system to examine whether HET has 5-HT₂CR blocking activity or not. Surprisingly, the results showed that HET has an agonistic, not an antagonistic, effect on 5-HT₂CR. Both its potency and efficacy seem to be very strong. Only $5 \mu\text{g/ml}$ as estimated galenicals evoked a strong inward current of almost the same intensity as that evoked by $10 \mu\text{M}$ 5-HT. If one pure compound in the extract evoked the current, the content was 1 % and the molecular weight was 200 (about the same as 5-HT), the $5 \mu\text{g/ml}$ corresponds to only $0.25 \mu\text{M}$ of the

pure compound. Furthermore, a higher concentration of HET evoked a much more intense current (data not shown). In contrast, 5-HT showed the maximum intensity at $10 \mu\text{M}$, and even at $1 \mu\text{M}$ almost the same intensity was shown. It is suggestive that HET includes strong 5-HT₂CR agonistic agent(s). There are several possibilities about the kind of agents involved in the HET-evoked current; 1) only one pure compound or multiple compounds with the same character are involved in the response and 2) some regulatory compounds are present in HET, like glycine in the case of the NMDA-type of glutamate current. Another interesting possibility about the origin of the compound(s) which is/are involved in the current is that one or more compounds originated from one or more galenicals react to make new compound(s) during the extraction step of boiling for 60 ~90 min. Further investigations will be necessary to test this possibility.

HET acts as an agonist for 5-HT₂CR in *Xenopus* oocytes, while imipramine has an antagonistic profile. Both HET and imipramine, however, enhanced the mRNA levels of 5-HT₂CR. In our previous paper we speculated that long-term occupation of 5-HT₂CR by antidepressant as an antagonist enhanced the 5-HT₂CR synthesis to compensate for the decreased receptor function. We also speculated that since 5-HT₂CR acts as a transformation factor in itself²³⁾ the increased 5-HT₂CR may regulate expression of other genes such as those encoding anti-stress proteins. What kinds of mechanisms are involved in agonist-, such as this HET, induced elevation of 5-HT₂CR mRNA level? The metabotropic response like that evoked by 5-HT in *Xenopus* oocytes has been reported to be desensitized by repeated application of agonist and by protein kinase C stimulation, which participates in this desensitization step.²⁴⁾ It has been reported that administration of a typical 5-HT₂CR agonist, *m*-chlorophenylpiperazine (mCPP), generates the desensitization of 5-HT₂CR agonist-induced hypothermia and elevation of plasma adrenocorticotrophic hormone (ACTH) level in rats^{25,26)} and humans.²⁷⁾ These results suggest that repeated administration of agonist may also inhibit the 5-HT₂CR function, apparently by desensitization. That may be the reason that HET elevated the 5-HT₂CR mRNA

levels as well as imipramine.

In contrast to the low concentration of HET required in the oocyte experiment, a relatively high dose was necessary to achieve the difference in mRNA expression. The dose of 5.7 g/kg p.o. as estimated galenicals is about 10-fold higher than the clinical dose in humans. At 0.57 g/kg which is almost the same dose used clinically, there was no significant effect on 5-HT₂CR mRNA levels. Around a 10-fold higher dose of imipramine (20 mg/kg p.o.), however, was also necessary to elevate the mRNA. Our previous results in time-course experiments revealed that 4 days of treatment with imipramine was enough to induce maximal expression of 5-HT₂CR mRNA at 20 mg/kg p.o.. Therefore, we also used these conditions in the HET experiment. However, 4 days of treatment seems to be shorter than required for therapeutic effects. If we use a similar dose clinically, longer-term treatment with HET or imipramine may be required to achieve therapeutic effects. Metabolic effects, including differences between rats and humans, and absorption, especially through the blood brain barrier, must also be considered. Furthermore, will also be of interest to see what kinds of receptors are involved in the ionotropic response of HET in *Xenopus* oocytes.

In conclusion, this report has clarified that HET includes factors which strongly stimulate 5-HT₂CR, and HET enhances 5-HT₂C mRNA levels as do antidepressants such as imipramine, which has a 5-HT₂CR antagonistic effect.

和文抄録

補中益気湯の“益気”作用機序を明らかにする目的で、熱水抽出エキスのセロトニン 2C サブタイプ受容体 (5-HT₂CR) 応答への作用を検討した。補中益気湯 5.7 g/kg (生薬換算量) およびイミプラミン 20 mg/kg を 1 日 1 回 4 日間ラットに経口投与したところ、脳内 5-HT₂CR mRNA 量が有意に増大した。ラット脳 mRNA 移入アフリカツメガエル卵母細胞再構成系において、補中益気湯はわずか 5 µg/ml (生薬換算量) の添加で、代謝性とイオンチャネル性の二種の強い内向き電流応答を示した。そのうち代謝性の応答は 5-HT₂CR 遮断薬である 10 µM ミアンセリンの前処置で完全に消失した。以上の結果より、補中益気湯は 5-HT₂CR を賦活化する作用を有し、それを介して抗うつ薬様効果がもたらされるものと

と推定された。

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