分担研究報告書

統合失調症に有効な抑肝散構成成分の薬理解析と単一成分固定

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研究要旨

認知症に伴う行動心理学的症状（BPSD）の治療に対して抑肝散が効果を示すことが報告されている。一方、BPSDの治療には非定型抗精神病薬が効果的であることが知られている。そこで非定型抗精神病薬と類似した成分が抑肝散の中にも含有されている可能性について検討した。その候補成分として抑肝散の構成生薬の釣藤鉤のインドールアルカロイド成分に着目し、中枢神経系に発現しているセロトニン受容体のサブタイプ（1A、2A、2C、7）に対してどの様に作用するかを細胞内Ca^{2+}イメージング法を用いて解析した。これらのセロトニン受容体はそれぞれ異なるGタンパク質と共役するため、選択的に共役するG蛋白とGα16蛋白との間でキメラ蛋白を作成することにより、全ての種類のG蛋白質共役型セロトニン受容体のシグナルをPI turnover系に集約し、Ca^{2+}イメージング法で解析を行った。その結果、釣藤鉤由来のインドールアルカロイドの１成分が5-HT1A受容体に対してはアゴニストとして、5-HT2A受容体、5-HT2C受容体、5-HT7受容体に対してはアンタゴニストとして働くことが分かった。この特徴は、第３世代の非定型抗精神病薬のアリピプラゾールのセロトニン受容体に対する作用の特徴と類似点が多く、統合失調症に有効な成分が抑肝散に含まれている可能性を示唆するものである。

A．研究目的

認知症に伴う行動心理学的症状（BPSD）の治療に対して抑肝散が効果を示すことが報告されている。BPSDに対して抑肝散は服用後比較的早期に効果があることから、少なくともこの効果には神経伝達機構が直接関与している可能性が高い。一方、BPSDの治療にはリスペリドンなどの非定型抗精神病薬が効果的であることが知られている。これらの事実から考え合わせると、非定型抗精神病薬と類似した成分が抑肝散の中にも含有されている可能性があり、抑肝散の構成成分の中に統合失調症に有効な成分が含まれている可能性がある。非定型抗精神病薬の作用機序としてドーパミンD2受容体以外にも多種類のセロトニン受容体サブタイプに同時に作用することが知られている。そこで本研究では抑肝散の中のアルカロイド成分が、各種セロトニン受容体サブタイプに対してどの様な影響を与えるかを解析する。

B．研究方法

抑肝散やそのアルカロイド成分が、中枢神経系に発現しているタイプのセロトニン受容体（1A、2A、2C、7）に対してどの様に作用するかを細胞内Ca^{2+}イメージング法を用いて解析した。これらのセロトニン受容体はそれぞれ異なるG蛋白と共役するため、セカンドメッセンジャーも異なり、単純にHEK細胞に各タイプのセロトニン受容体を発現させただけでは、Ca^{2+}イメージング法で反応が得られない。本研究ではこの点を解消するため、それぞれのタイプのセロトニン受容体と選択的に共役するGα蛋白とGα16蛋白との間でキメラ蛋白を作成することにより、全ての種類のGタンパク質共役型セロトニン受容体のシグナル伝達をPI turnover系に集約し、Ca^{2+}イメージング法で解析を行った。また、実験に用いる各種セロトニン受容体は、RT-PCR法により脳組織から単離した。本研究では抑肝散の構成生薬の釣藤鉤のアルカロイド成分であるガイソシジンメチルエーテル、リンコフィリン、イソリンコフィリン、ヒルスチン、ヒルステイン、コリノキセイン、イソコリノキセインなどがセロトニン受容体に与える影響について検討した。

C．研究結果

HEK293 細胞に 5-HT1A 受容体、5-HT2A 受容体、5-HT2C 受容体の cDNA を強制発現させ、カルシウムイメージング法で薬剤の感受性を検討した。特に

5-HT1A 受容体は元来 Gi 蛋白と共役し cAMP を減少させるため、そのままではカルシウムイメージング法による測定はできないので、G16/o と G16/i3 のキメラ G 蛋白を 5-HT1A 受容体と共役させることにより、カルシウムイメージング法での測定を可能にした。

これらの方法を用いて検討した結果、投与した多種のアルカロイドの中で一つの成分のみが、セロトニン受容体に影響を与えることが分かった。このアルカロイドは5-HT1A受容体、5-HT2A受容体、5-HT2C 受容体、5-HT7 受容体にそれぞれ作用するが、5-HT1A 受容体に対しては、アゴニストとして働いた。5-HT2A 受容体に対しては、主にアンタゴニストとして働き、5-HT2C 受容体や 5-HT7 受容体に対してもアンタゴニストとして働いた。

D．考察

Gタンパク質共役型のセロトニン受容体のサブタイプは多様性に富み、5-HT1受容体はGiタンパク質と共役し細胞内のcAMPを減少させ、5-HT2受容体はGqタンパク質と共役し細胞内のカルシウムを上昇させ、5-HT4～7受容体はGsタンパク質と共役し細胞内のcAMPを上昇させると考えられているが、本研究ではG16/oやG16/i3などのキメラGタンパク質を各種セロトニン受容体とHEK細胞に共発現させることにより、全てのGタンパク質共役型のセロトニン受容体をカルシウムイメージング法でアッセイできるように条件を調整した。

一方、抑肝散の構成生薬の一つである釣藤鉤のインドールアルカロイド成分がセロトニンと類似構造を共有していることに着目し、ガイソシジンメチルエーテル、リンコフィリン、イソリンコフィリン、ヒルスチン、ヒルステイン、コリノキセイン、イソコリノキセインなどがセロトニン受容体の各種サブタイプに親和性があるかどうかを検討した。その結果、その中の一成分のみが5-HT1A受容体に対してアゴニスト、5-HT2A受容体と5-HT2C受容体と5-HT7受容体に対してアンタゴニスト活性を示した。この特徴は、第３世代の非定型抗精神病薬のアリピプラゾールのセロトニン受容体に対する作用の特徴と類似点が多く認められた。このことは、統合失調症に有効な成分が抑肝散に含まれている可能性を示唆するものである。

E．結論

非定型抗精神病薬と類似した成分が抑肝散の中にも含有されている可能性について検討した。その候補成分として抑肝散の構成生薬の釣藤鉤のインドールアルカロイド成分に着目し、脳に発現しているセロトニン受容体のサブタイプ（1A、2A、2C、7）に対してどの様に影響を与えるかを細胞内 Ca^{2+}イメージング法を用いて解析した。セロトニン受容体の各種サブタイプはそれぞれ異なるGタンパク質と共役するため、本研究ではキメラGタンパク質を作成することにより、全ての種類のGPCR型のセロトニン受容体のサブタイプをCa^{2+}イメージング法で解析した。その結果、釣藤鉤由来のインドールアルカロイドの１成分が5-HT1A受容体に対してはアゴニストとして、5-HT2A受容体、5-HT2C受容体、5-HT7受容体に対してはアンタゴニストとして働くことが分かった。この特徴は、第３世代の非定型抗精神病薬のアリピプラゾールのセロトニン受容体に対する作用の特徴と類似点が多く、統合失調症に有効な成分が抑肝散に含まれている可能性を示唆するものである。

F．健康危険情報

なし

G. 研究発表

1. 論文発表

総説

島田昌一、石田雄介、鵜川眞也、植田高史、
キメラGタンパク質を用いたGタンパク質共役型受容体（GPCR）のアッセイ、脳２１、14:68-73、2011.

2. 学会発表

Shoichi Shimada, Yusuke Ishida, Shinya Ugawa, Takashi Ueda, The effect of Uncaria alkaloids on serotonin receptors, Conference on the Recent Development in Chinese Herbal Medicine 2010, Singapore.

H. 知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得

統合失調症の予防又は治療薬
（特願2010-002364）出願中

2. 実用新案登録

なし

3. その他

なし

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Hiratsuka T, Matsuzaki S, Miyata S, Kinoshita M, Kakehi K, Nishida S, Katayama T, Tohyama M.	Yokukansan inhibits neuronal death during ER stress by regulating the unfolded protein response.	PLoS One.	5(10)	e13280	2010
島田昌一、石田雄介、鵜川眞也、植田高史	キメラGタンパク質を用いたGタンパク質共役型受容体（GPCR）の	脳２１	14	68-73	2011

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Yokukansan Inhibits Neuronal Death during ER Stress by Regulating the Unfolded Protein Response

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Abstract

Background: Recently, several studies have reported Yokukansan (Tsumura TJ-54), a traditional Japanese medicine, as a potential new drug for the treatment of Alzheimer's disease (AD). Endoplasmic reticulum (ER) stress is known to play an important role in the pathogenesis of AD, particularly in neuronal death. Therefore, we examined the effect of Yokukansan on ER stress-induced neurotoxicity and on familial AD-linked presenilin-1 mutation-associated cell death.

Methods: We employed the WST-1 assay and monitored morphological changes to evaluate cell viability following Yokukansan treatment or treatment with its components. Western blotting and PCR were used to observe the expression levels of GRP78/BiP, caspase-4 and C/EBP homologous protein.

Results: Yokukansan inhibited neuronal death during ER stress, with Cnidii Rhizoma (Senkyu), a component of Yokukansan, being particularly effective. We also showed that Yokukansan and Senkyu affect the unfolded protein response following ER stress and that these drugs inhibit the activation of caspase-4, resulting in the inhibition of ER stress-induced neuronal death. Furthermore, we found that the protective effect of Yokukansan and Senkyu against ER stress could be attributed to the ferulic acid content of these two drugs.

Conclusions: Our results indicate that Yokukansan, Senkyu and ferulic acid are protective against ER stress-induced neuronal cell death and may provide a possible new treatment for AD.

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☯ These authors contributed equally to this work.

Introduction

Yokukansan (Tsumura TJ-54), a traditional Japanese medicine, has traditionally been administered to patients who show symptoms such as nervousness, short-temperedness, irritability, sleeplessness, twitching of the eyelids and shaking of the limbs. It has also been administered to infants who suffer from night crying, restlessness and convulsions. Recently, several clinical reports have shown that Yokukansan is effective against the Behavioral and Psychological Symptoms of Dementia (BPSD) and improves daily living of patients [1–3]. Thus, Yokukansan has been suggested as a possible new candidate for treating Alzheimer's disease (AD). However, no basic research on the clinical effects of Yokukansan has been conducted.

Many reports have suggested that endoplasmic reticulum (ER) stress is involved in the pathogenesis of AD, with several studies showing that the amyloid β protein, which is abundant in the AD brain, induces ER stress [4–6]. Previous studies from our laboratory have shown that the familial AD (FAD)-linked presenilin-1 (PS1) mutation increases the susceptibility to ER stress and that the presenilin-2 (PS2) splice variant (PS2V), observed in the sporadic form of AD, also increases the risk of ER stress [7–12]. These results suggest that ER stress is involved in the pathogenesis of AD.

ER stress activates both the survival and apoptotic pathways. In the survival pathway, ER stress induces the transcription of genes encoding for the ER-resident chaperones such as GRP78/Bip, GRP94 and protein disulfide isomerase (PDI), which facilitate protein folding. This induction system is termed the 'unfolded-protein response (UPR) [13–16]. By contrast, the representative gene C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage-inducible gene 153 (GADD153), is induced in the apoptotic pathway [16–17]. In addition, we have revealed the involvement of caspase-4, a protease that is

specifically induced by ER stress in humans and may be involved in the pathogenesis of AD [18]. The familial AD-linked PS1 mutation accelerates the cleavage of caspase-4, which in turn activates caspase-3 and caspase-9 without involving the cytochrome-c pathway [19]. These results suggest that the initiation of caspase-4 cleavage is one of the key events for the pathogenesis of AD.

In this report, we studied the effect of Yokukansan on ER stress-induced neurotoxicity and on FAD-linked PS1 mutation (ΔE9) associated cell death. We determined that upregulation of GRP78/Bip expression by Yokukansan, as well as the inhibition of CHOP induction, results in a reduction of ER stress-induced cell death and FAD-linked associated cell death. In addition, we showed that Yokukansan inhibits the activation of caspase-4. Furthermore, we exhibited that the effects of Yokukansan could be attributed to the function of Cnidii Rhizoma (Senkyu), a component of Yokukansan. We determined that the ferulic acid contained in Senkyu plays an important role for the protective function of Yokukansan or Senkyu. These results show that Yokukansan, Senkyu or ferulic acid alone could be a potential treatment for AD and our findings cast new light on the development of new therapies for AD.

Results

Yokukansan reduces ER stress-induced neuronal cell death

We examined the effects of Yokukansan on neuronal cell death caused by several stresses using the mouse neuroblastoma cell line, Neuro2a (N_2a). Thapsigargin (TG) and hypoxia were used as ER stress inducers and staurosporine (STS) was used as a mitochondrial stress inducer. Yokukansan significantly decreased the cell death caused by TG and hypoxia (Figure 1A and 1B), but did not protect against STS treatment (Figure 1B). These results indicate that Yokukansan is effective against ER stress-induced neuronal toxicity that involves impairment of calcium homeostasis, but not apoptotic stimuli that do not cause ER stress. Notably, as shown in Figure 1C, the protective effect of Yokukansan against ER stress-induced cell death is proportional to the concentration of Yokukansan used. However, a high dose of Yokukansan showed some toxicity.

Cnidii Rhizoma (Senkyu), a component of Yokukansan, has a potent protective effect against ER stress-induced neuronal toxicity

To determine which component of Yokukansan plays a key role in inducing the protective effect against neuronal cell death caused by ER stress, we examined the effect of each of the 7 components of Yokukansan on neuronal death caused by TG using the N_2a cell line. Cnidii Rhizoma (Senkyu), Hoelen (Bukuryo) and Angelicae Radix (Toki) significantly decreased cell death caused by TG, and Bupleuri Radix (Saiko) showed some neuroprotective effect against TG ($P = 0.054$) (Figure 2A). However, the other components of Yokukansan (Atractylodis Lanceae Rhizoma (Soujutsu), Glycyrrhizae Radix (Kanzo) and Uncariae Uncis Cum Ramulus (Chotoko)) failed to inhibit cell death caused by TG (Figure 2A and data not shown). When using the human neuroblastoma cell line SK-N-SH, Senkyu and Saiko induced a significant reduction in neuronal death caused by TG (Figure 2B). However, the other components of Yokukansan did not show any neuroprotective effect against TG. Senkyu was the most potent inhibitor of neuronal cell death following TG-induced toxicity in both N_2a and SK-N-SH cells. Therefore, our subsequent analysis focused on the effect of Senkyu on neuronal death caused by ER stress.

Senkyu and Yokukansan reduce neuronal toxicity caused by the FAD-linked PS1 mutation

Previously, we demonstrated that mutations in PS1 increase vulnerability to ER stress by altering the signaling pathway. We stably transfected SK-N-SH cells with complementary DNA constructs encoding for wild-type PS1 and PS1 with a deletion of exon 9 (ΔE9), which is one of the FAD-linked mutations. As shown in Figure 3A, the addition of TG induced a greater cell death rate in cells expressing the mutant PS1 when compared with cells expressing the wild-type protein. In addition, pretreatment with Yokukansan reduced cell death to wild type levels in TG treated cells expressing ΔE9. Moreover, Senkyu inhibited cell toxicity following TG treatment in a concentration-dependent manner, resulting in levels similar to those seen in dimethyl sulfoxide (DMSO)-treated cells (Figure 3B). These results indicate

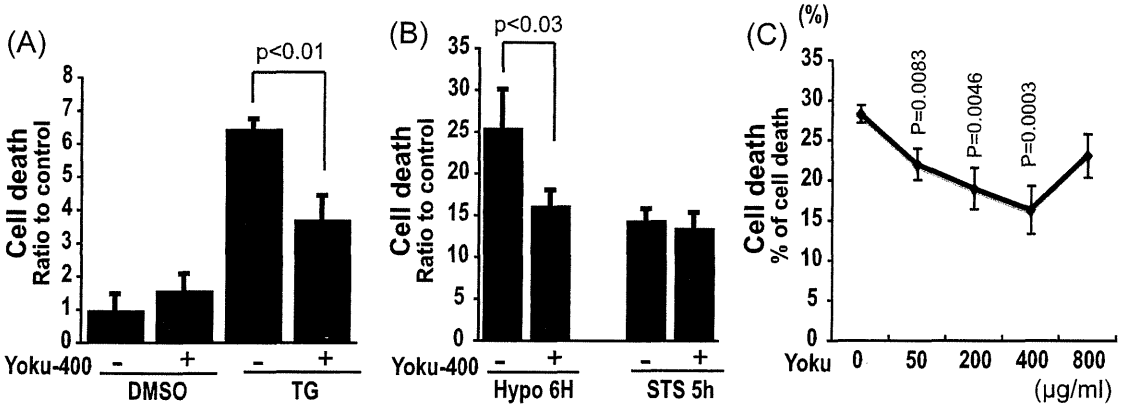

Figure 1. Yokukansan reduces ER stress-induced neuronal cell death. Cell toxicity in N_2a cells was measured based on morphological changes. Quantitative data are expressed as the mean ± SEM for at least three independent experiments. The P value was compared with the control and calculated by Student's T test. (A) Cell death was measured 6.5 h after 1 μM TG or DMSO (control) exposure with or without 1.5 h of pretreatment with 400 μg/ml Yokukansan. (B) Cell death was measured 6 h after hypoxia exposure and 5 h after 0.1 μM STS exposure with or without 1.5 h of pretreatment with 400 μg/ml Yokukansan. Non treated cells were used as the control. (C) Cell death was measured 6.5 h after 1 μM TG exposure with 1.5 h of pretreatment with the indicated concentration of Yokukansan.
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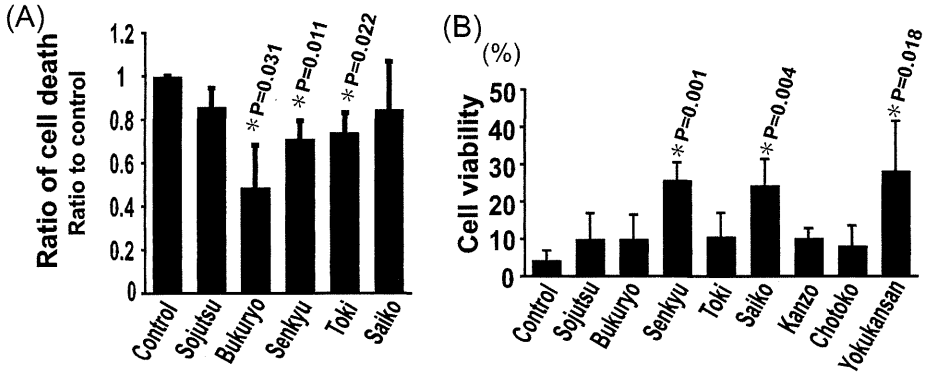

Figure 2. Cnidii Rhizoma (Senkyu), a component of Yokukansan, has potent protective effects against ER stress- induced neuronal toxicity. N_2a cell toxicity and SK-N-SH cell viability was measured based on morphological changes and the WST-1 assay, respectively. Quantitative data are expressed as the mean ± SEM for at least three independent experiments. The P value was compared with the control and calculated by Student's T test. (A) Cell death was measured 20 h after 1 μM TG exposure with 1.5 h pretreatment with 200 μg/ml of each of the indicated components of Yokukansan. (B) Cell viability was measured 3 h after 3 μM TG exposure with 1.5 h pretreatment with 200 μg/ml of each of the indicated components of Yokukansan. Cells incubated with TG without any pretreatment were used as a control.
doi:10.1371/journal.pone.0013280.g002

that Yokukansan may be able to rescue cells from ER stress caused by the AD-linked mutation through the effect of Senkyu.

Senkyu and Yokukansan reduce the vulnerability to ER stress by altering the unfolded protein response (UPR) signaling pathway

Normal cells respond to ER stress by increasing the transcription of genes encoding for the ER-resident chaperons such as GRP78/Bip, GRP94 and PDI, which facilitate protein folding (unfolded protein response). An increase in GRP78/Bip expression leads to cell survival. However, ER stress can also induce CHOP expression and activation of the JNK pathway, which induce cell death. Therefore, to determine the molecular mechanism of the neuroprotective effect of Senkyu and Yokukansan against ER stress, we examined the basal expression levels of GRP78/Bip and the expression levels of mRNA encoding for CHOP following TG toxicity after Senkyu or Yokukansan treatment. Both Senkyu and Yokukansan upregulated GRP78/Bip expression when compared with the no treatment control (Figures 4A and 4B). By contrast, both Senkyu and Yokukansan treatment significantly reduced CHOP expression when compared with cells treated with TG alone (Figures 4C and 4D).

Senkyu and Yokukansan inhibit the activation of caspase-4, an ER stress-specific apoptotic protease

Caspase-4 has been shown to be involved in ER stress-induced neuronal cell death and in the pathogenesis of AD [18,19]. We have shown that Yokukansan and Senkyu reduce ER stress-induced neuronal cell death (Figures 1, 2, 3 and 6). Therefore, we examined the effect of Yokukansan and Senkyu on the activation of caspase-4. As shown in Figures 5A and 5B, both Senkyu and Yokukansan inhibited the cleavage of caspase-4. Thus, the protective effect of Yokukansan and Senkyu against ER stress can be partially attributed to the inactivation of caspase-4 as well as regulation of the UPR.

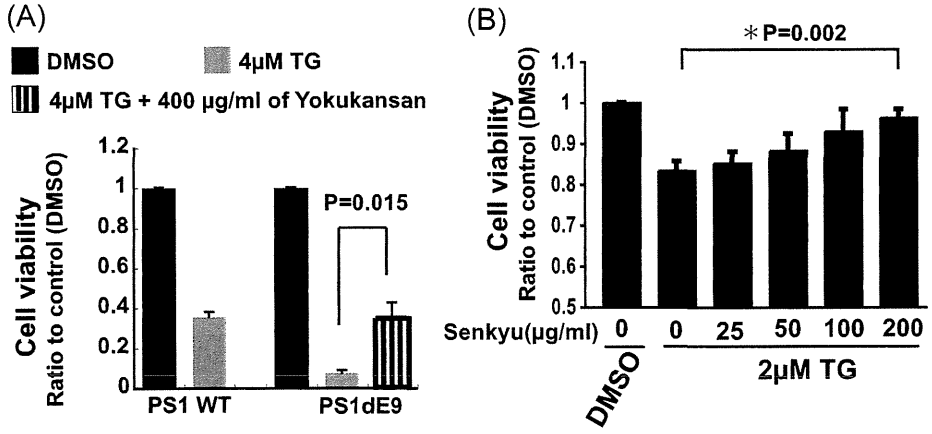

Figure 3. Senkyu and Yokukansan reduce neuronal toxicity caused by the FAD linked PS1 mutation. Cell viability of SK-N-SH cells expressing PS1 WT or PS1ΔE9 was measured using the WST-1 assay. Quantitative data are expressed as the mean ± SEM for at least three independent experiments. The P value was compared with the control (DMSO treated cells) and calculated by Student's T test. (A) Cell viability was measured 3 h after 4 μM TG exposure with or without a 1.5 h pretreatment with 400 μg/ml of Yokukansan. (B) Cell viability of cells expressing PS1ΔE9 was measured 3 h after 2 μM TG exposure with a 1.5 h pretreatment with Senkyu at the indicated concentrations (0, 25, 50, 100, 200 μg/ml).
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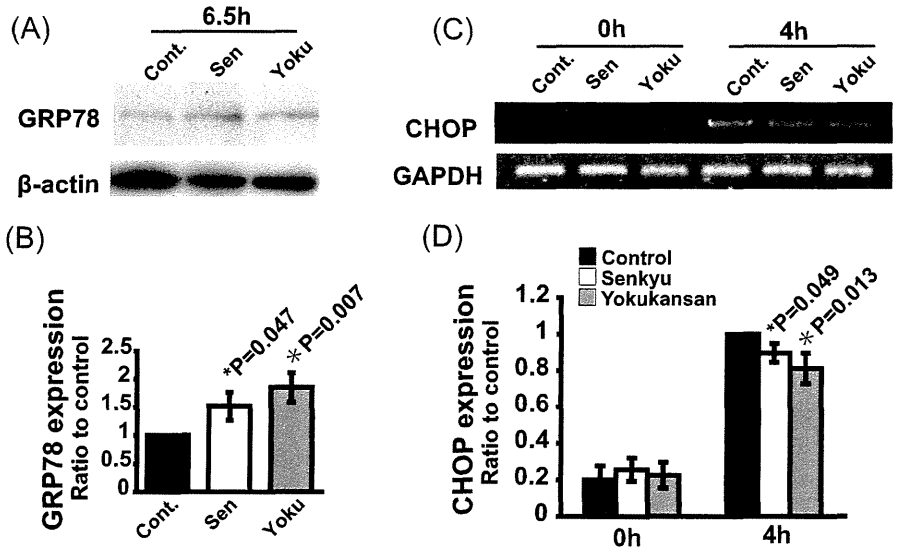

Figure 4. Senkyu and Yokukansan reduce susceptibility to ER stress by altering the unfolded protein response (UPR) signaling pathway. (A and B) SK-N-SH cells were treated with Senkyu or Yokukansan (200 μg/ml) for 6.5 h. Cells were lysed and western blot analysis was performed using an anti-Bip or anti-b-actin primary antibody (A). Quantitative data were obtained by densitometry of the bands. Data are expressed as the mean ± SEM for at least three independent experiments (shown as a ratio of the control). The P value was compared with the control and calculated by Student's T test (B). (C and D) SK-N-SH cells were treated with 1 μM TG with or without a 1.5 h pretreatment with Senkyu or Yokukansan (200 μg/ml). The expression of CHOP mRNA and GAPDH mRNA were detected by RT-PCR (C). Quantitative data were obtained by densitometry of the bands. Data are expressed as the mean ± SEM for at least three independent experiments (shown as a ratio of the control). The P value was compared with the control and calculated by Student's T test (D).
doi:10.1371/journal.pone.0013280.g004

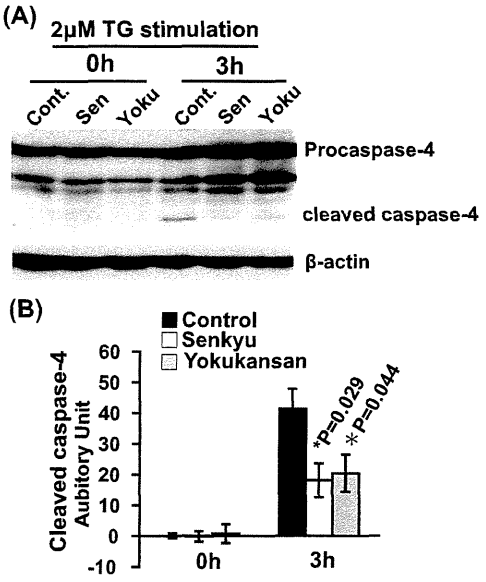

Figure 5. Senkyu and Yokukansan inhibit the activation of caspase-4. (A and B) SK-N-SH cells were treated with 2 μM TG for 3 h with or without a 1.5 h pretreatment with Senkyu (200 μg/ml) or Yokukansan (200 μg/ml). Cells were lysed and western blot analysis was performed using an anti-caspase-4/TX or anti-b-actin antibody (A). Quantitative data were obtained by densitometry of the bands. Data are expressed as the mean ± SEM for at least three independent experiments (shown as a ratio of the control). The P value was compared with the control and calculated by Student's T test (B).
doi:10.1371/journal.pone.0013280.g005

Ferulic acid plays an important role in cell survival during ER stress

Our results indicate that Yokukansan induces resistance against ER stress by regulating the UPR and apoptotic pathway, particularly following Senkyu treatment, one of the components of Yokukansan. To confirm our results, we examined the effect of Senkyu-free Yokukansan, which contains all components except Senkyu. The Senkyu-free Yokukansan did not improve cell viability (Figure 6). These results show that Senkyu is important for survival during ER stress-induced toxicity. To determine how Senkyu induces its neuroprotective effect, we screened the contents of Senkyu as shown in Figure S1. As a result, we identified two potent candidates, ferulic acid and coniferyl ferulate (Figure S2). Ferulic acid, a plant constituent, has been reported to be a strong free radical scavenger with an antioxidant capacity [20]. In addition, ferulic acid has many pharmacological effects such as anti-inflammatory, anticancer, anti-diabetic, anti-atherogenic and neuroprotective [21–25]. Furthermore, ferulic acid has been reported to be protective against amyloid β protein toxicity [26,27]. Thus, we focused on ferulic acid. To elucidate the function of ferulic acid, we monitored the effect of ferulic acid pretreatment on cell viability following ER stress. Ferulic acid was neuroprotective against ER stress in a concentration-dependent manner and provided similar protection to that of Yokukansan- or Senkyu-pretreated cells (Figure 6). In addition, we confirmed this result by treating cells with a mixture of ferulic acid and Senkyu free Yokukansan (Figure 6).

Effect of Yokukansan, Senkyu and Ferulic acid on the UPR

We have shown that induction of GRP78/Bip and reduction of CHOP following pretreatment with Yokukansan or Senkyu reduces cell toxicity caused by ER stress. Therefore, we examined

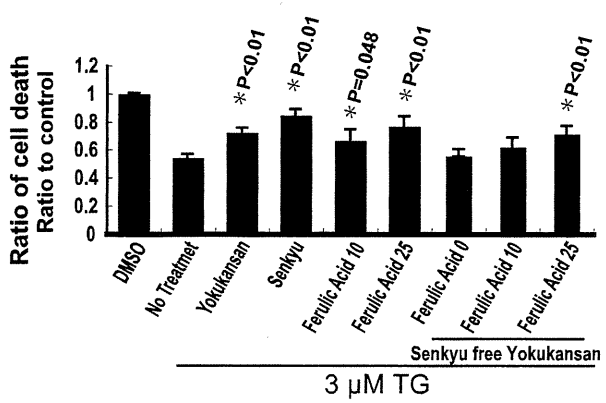

Figure 6. Ferulic acid, a component of Cnidii Rhizoma (Senkyu), has potent protective effects against ER stress-induced neuronal toxicity. Cell viability of SK-N-SH cells was measured using the WST-1 assay. Quantitative data are expressed as the mean ± SEM for at least three independent experiments. The P value was compared with the control (DMSO treated cell) and calculated by Student's T test. Cell viability was measured 3 h after 3 μM TG exposure with or without a 2 h pretreatment with 200 μg/ml of Yokukansan, Senkyu-free Yokukansan or Senkyu, with or without ferulic acid at the indicated concentrations (0, 10, 25 μg/ml). Cells incubated with TG without any pretreatment were used as a control.
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the effect of ferulic acid on the expression levels of GRP78/Bip and CHOP. Pretreatment with ferulic acid increased the mRNA expression level of GRP78/Bip. A similar result was observed following pretreatment with Yokukansan or Senkyu (Figures 4A, B and 7A). In addition, CHOP induction, caused by ER stress, was reduced following pretreatment with ferulic acid, as was seen following pretreatment with Yokukansan or Senkyu (Figure 4C, D and 7B). These results suggest the involvement of ferulic acid in the regulation of the UPR signaling pathway.

Discussion

Recently, several clinicians have observed the effectiveness of Yokukansan, a traditional Japanese medicine, in the treatment of the BPSD and in cognitive impairment of AD [1–3]. However, the molecular mechanism remains unclear. In this study, we examined the effect of Yokukansan on cell death caused by TG, STS or hypoxia. Yokukansan reduced the cell death caused by TG and hypoxia, both of which induce ER stress via abnormal Ca^{2+} homeostasis, but were unable to protect against neural toxicity caused by STS, a mitochondrial stress inducer. These results suggest that Yokukansan may not be effective against mitochondrial stress related toxicity, but on ER stress related cell toxicity (Figure 1). In addition, recent studies have reported that Yokukansan has preventive or inhibitive effects against the development of memory disturbance, BPSD-like behaviors and neurodegeneration, all of which are observed in thiamine deficient rodents because of ER stress due to thiamine deficicency [28–30]. These reports support our hypothesis that Yokukansan may play an important role against ER stress.

Yokukansan consists of several components. Therefore, it was important to determine which components were effective against ER stress. Thus, we investigated the effect of each component on TG induced cell death. As shown in Figure 2, Bukuryo and Toki reduced cell death in N_2a cells, but not in SK-N-SH cells; Saiko inhibited cell death in SK-N-SH cells, but not that of N_2a cells; only Senkyu rescued both N_2a and SK-N-SH cells from TG-induced cell toxicity. Such differences in effects on the cell death observed following Bukuryo, Toki and Saiko pretreatment may be due to differences in the exposure time and concentration of TG or differences between the cell lines. Nevertheless, we focused on Senkyu because it was the only component that reduced cell death under both conditions.

As mentioned previously, our results provide strong evidence that Senkyu, a component of Yokukansan, protects against ER stress-induced neuronal cell death, particularly against ER-stress caused by intracellular calcium homeostasis abnormalities (Figure 1, 2, 3 and 6). In addition, pretreatment with Senkyu upregulated GRP78/Bip expression and down-regulated CHOP expression caused by ER stress (Figure 4). GRP78/Bip is known to protect cells from cell death caused by ER stress [13–16], while CHOP induces cell death during ER stress [16,17]. Thus, Senkyu may inhibit ER stress-induced neuronal cell death via regulation of the UPR and the apoptotic cascade. We also examined the effect of Senkyu and Yokukansan on neuronal cell death caused by the down-regulation of the UPR signaling pathway as a result of FAD-linked PS1 mutations [7,8]. As shown in Figure 3, both Yokukansan and Senkyu improved the viability of cells under TG stimulation. In addition, we elucidated that Yokukansan and

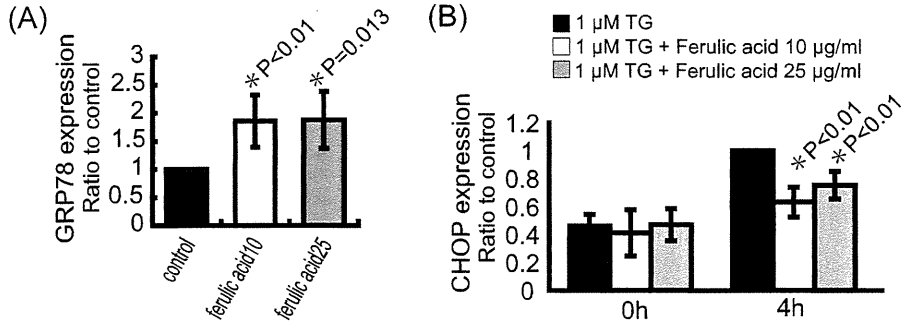

Figure 7. Ferulic acid, Senkyu and Yokukansan reduce susceptibility to ER stress by altering the unfolded protein response (UPR) signaling pathway. (A) SK-N-SH cells were treated with ferulic acid at the indicated concentrations (0, 10, 25 μg/ml) for 2 h. (B) SK-N-SH cells were treated with 1 μM TG with or without a 2 h pretreatment with ferulic acid at the indicated concentrations (0, 10, 25 μg/ml). (A, B) The expression of GRP78/Bip (A), CHOP (B) and GAPDH (A, B) mRNA were detected by RT-PCR. Quantitative data were obtained by densitometry of the bands. Data are expressed as the mean ± SEM for at least three independent experiments (shown as a ratio of the control). The P value was compared with the control and calculated by Student's T test.
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Senkyu inhibited the activation of caspase-4 observed under ER stress (Figure 5). Thus, the reduction of ER stress-induced cell death could be attributed to the inactivation of caspase-4 and regulation of the UPR signaling pathway. These findings suggest that Yokukansan or Senkyu alone may be an effective candidate for the treatment of AD.

Based on these findings, we proceeded to screen the contents of Senkyu and Yokukansan to determine their function. As a result, we found ferulic acid, which has been shown to be protective against amyloid β toxicity and oxidative stress [26,27]. As shown in Figure 6, similar to Yokukansan and Senkyu, ferulic acid reduced cell death following ER stress. Furthermore, ferulic acid regulated the expression levels of GRP78/Bip and CHOP (Figure 7). These findings indicate that the protective effects of Yokukansan and Senkyu are due to ferulic acid.

We also observed that long term treatment or a high dose of Yokukansan had a neurotoxic effect on cultured neuronal cells (Figure 1C) and that some components of Yokukansan (Kanzo and Chotoko) also had the same effect with respect to neurotoxicity (Figure S3). However, longer exposure to Senkyu or ferulic acid did not induce neurotoxicity (data not shown). These data indicate that Senkyu or ferulic acid would be more clinically advantageous in terms of safety.

Several studies have reported that Chotosan, another traditional Japanese medicine, is also effective against amyloid β toxicity [31–33]. Similar to Yokukansan, the components of Chotosan, which include Chotoko, Bukuryo and Kanzo, activate neprilysin and insulin degrading enzyme (IDE), both of which are proteases of the amyloid β protein [33]. Given that Chotosan does not contain Senkyu, it is possible that some of the neuroprotective effects seen with Yokukansan following amyloid β toxicity could be attributed to the other components of Yokukansan. However, the presence of ferulic acid in Yokukansan does partially explain the neuroprotective effect observed following amyloid β protein toxicity [26–27]. Considering the following results: 1: A high dose of Yokukansan causes neural toxicity, but a high dose of Senkyu or ferulic acid does not show any toxicity (Figures 1C and 3B, Figure S3 and data not shown), 2: Senkyu reduces cell death following PS1 mutations [7,8] (Figure 3), 3: Senkyu and ferulic acid reduce cell death caused by Ca^{2+}-related ER stress, which could be induced by amyloid β protein [34,35] (Figures 2 and 6), 4: Senkyu inhibits the activation of caspase-4 under ER stress, which could lead to neural death [18,19] (Figure 5), 5: Senkyu and Ferulic acid regulate the UPR signaling pathway, which is activated by amyloid β protein [36] (Figures 4 and 7), 6: Ferulic acid prevents cell death due to amyloid β protein toxicity [27] and also induces resistance to amyloid β1-42 toxicity in the brain [26]; Senkyu or ferulic acid alone may be suitable drugs for AD therapy because both medicines inhibit ER stress following amyloid β toxicity [7,8,18,19,34–36].

At present, the therapeutic drugs available for AD include cholinesterase inhibitors and NMDA-receptor antagonists. However, their therapeutic effect is not significant [37–40]. A number of trials to develop effective drugs for AD have been performed based upon the amyloid β hypothesis or tau hypothesis [41–46]. However, the development of a truly effective treatment for AD is far away.

It has been reported that neuronal death observed in AD is related to ER stress [4–12,18,19]. In this study, we used TG as an ER stressor. TG, a highly lipophilic sesquiterpene lactone, is broadly used as a selective inhibitor of sarcoplasmic reticulum calcium-ATPase (SERCA), which pumps calcium from the cytosol into the lumen of the ER in mammalian cells. TG-mediated irreversible inhibition of ER calcium-ATPases can also cause the induction of calcium leakage from the ER to the cytoplasm, further facilitating the depletion of calcium within the ER, resulting in an increase in cytoplasmic calcium levels [47]. Long-term elevation of intracellular calcium can induce ER stress due to misfolded protein accumulation [48,49]. Our results show that Senkyu and Yokukansan were protective against TG toxicity (Figures 1, 2, 3 and 6). This study has shown that ferulic acid, Senkyu and Yokukansan could be potential drugs for the treatment of AD and sheds some light for the development of new AD therapies.

Materials and Methods

Yokukansan, its components and Senkyu-free Yokukansan

Yokukansan (TJ-54) consists of Sojutsu (Atractylodes Lancea rhizome), Bukuyo (Hoelen), Senkyu (Cnidii Rizoma), Chotoko (Uncariae Uncis Cum Ramulus), Toki (Angelicae Radix), Saiko (Bupleuri Radix) and Kanzo (Glycyrrhizae Radix). Yokukansan is extracted from a mixture of dried plants as follows; 4 g of Sojutsu, 4 g of Bukuryo, 3 g of Senkyu, 3 g of Toki, 2 g of Saiko, 1.5 g of Kanzo and 3 g of Chotoko were added to 700 ml of distilled water and boiled for 1 hour, filtered, and then concentrated to 300 ml. On the other hand, to prepare Senkyu-free Yokukansan, the same extraction-method and amount of Soujutsu, Bukuyo, Chotoko, Toki, Saiko and Kanzo were used. Yokukansan, components of Yokukansan and Senkyu-free Yokukansan were kindly provided by Tsumura & Co. (Tokyo, Japan).

Chemicals and antibodies

We used the following antibodies: anti-Bip mAb (Cell Signaling Technology, Beverly, MA), anti-caspase-4/TX mAb (4B9; MBL International Corporation, Nagoya, Japan), monoclonal anti-β actin antibody (Chemicon, Temecula, CA) and HRP-conjugated anti-mouse IgG antibody (Cell Signaling Technology). The chemical reagents used in this experiment were thapsigargin (TG), staurosporine (STS) (Sigma-Aldrich, St. Louis, Mo) and ferulic acid (LKT Laboratories, Inc., St. Paul, MN).

Cell Culture

SK-N-SH human neuroblastoma cells were obtained from the Riken Cell Bank (Tsukuba, Japan). Neuro-2a mouse neuroblastoma cells (N_2a cells) were obtained from ATCC (Manassas, VA). Human neuroblastoma SK-N-SH cells and Mouse neuroblastoma N_2a cells were cultured in DMEM (Sigma) containing 10% (v/v) fetal bovine serum and incubated in a humidified chamber at 37°C with a 5% CO_2 atmosphere according to previous experiments [7–12]. SK-N-SH neuroblastoma cell lines stably expressing wild-type PS1 (PS1 WT cells) or PS1ΔE9 (PS1ΔE9 cells), which have been described previously [7], were cultured similarly to SK-N-SH cells.

Cell viability assay based on morphological changes

Cell toxicity in N_2a cells was measured on the basis of morphological changes observed by phase contrast microscopy or nuclear changes detected by fluorescence microscopy after co-staining cells with 10 μM Hoechst 33342 and 10 μM propidium iodide (PI). Hence, nuclear fragmentation was detected by Hoechst-positive staining and nuclear collapse was detected by PI-positive staining. Double positive cells were considered dead cells. Staining was measured independently in 4 fields and at least 300 cells were counted. Data are expressed as the mean ± SEM for at least three independent experiments.

Cell viability assay by WST-1 activity

SK-N-SH cells, PS1 WT cells, or PS1ΔE9 cells (3×10^3) were plated onto 96-well plates 36 h before cell viability was determined. Prior to performing the assay, cells were pretreated for 1.5 h with Yokukansan, or with every component of Yokukansan, at the indicated concentrations followed by the addition of each insult (TG or STS) for 3 h. Following insult exposure, cells were washed twice with phosphate buffered saline (PBS) and cultured with DMEM (D1145, SIGMA) and WST-1 mixed medium for 3 hours. WST-1 was measured at an absorption of λ450 nm - λ650 nm. Data are expressed as the mean ± SEM for at least three independent experiments.

Western blot analysis

Treated cells were washed twice with PBS, harvested and lysed in TNE buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA and 150 mM NaCl) containing 1% (v/v) NP-40 and protease inhibitor cocktail (Roche, Sydney, Australia). Equal amounts of protein were subjected to 12% (v/v) SDS-PAGE for GRP78/Bip, caspase-4 or β actin and transferred to PVDF membrane (Millipore, Bedford, MA). The membrane was blocked with 5% (w/v) skim milk and incubated with primary antibody, followed by incubation with an HRP-conjugated secondary antibody. Proteins were visualized with an ECL detection system (Amersham Biosciences, Piscataway, NJ).

Exposure to hypoxia

For the hypoxic insult, cells were exposed to hypoxia for 6 h using an incubator equipped with a hypoxic chamber that maintained a humidified atmosphere with low oxygen tension (8 Torr) as described previously [9,11,12].

RT-PCR

Total RNA was extracted from cultured SK-N-SH cells treated with the indicated reagents using the High Pure RNA Tissue Kit (Roche).The cDNA was synthesized from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Oligonucleotide sequences used for PCR were as follows; For GRP78/Bip, forward: 5′-agcctggcgacaagagtg-3′, reverse: 5′-tccttgggcagtattggatt-3′; For CHOP, forward: 5′-gcgcatgaaggagaaagaac-3′, reverse: 5′-ccaattgttcatgcttggtg-3′; For GAPDH, forward: 5′-ccactcctccacctttgacg-3′, reverse; 5′-caccctgttgctgtagccaa-3′.

Extraction of contents from Senkyu

Cnidii Rhizoma (Senkyu, 100 g) was powdered and extracted with hot water (500 mL, reflux, 1 h ×2). The water solution was concentrated under reduced pressure to obtain a water extract. The crude water-extract (100 mg) was dissolved in PBS (10 mL), and extracted for 10 min in an ultrasonic water bath. The extraction was repeated twice. The extracted solutions were combined and centrifuged at 10000 rpm for 10 min. The supernatant was recovered and diluted to the appropriate concentration with assay medium.

Supporting Information

Figure S1 Screening of contents of Senkyu. Extracts of Senkyu were divided into #1-6 fractions. We preliminary checked the effect of each fraction (#1-6) against the neural toxicity of TG by using SK-N-SH cells. Fractions #1-5 did not show any protective effect under TG stimulation, but fraction #6 showed a protective effect against TG-induced ER stress.
Found at: doi:10.1371/journal.pone.0013280.s001 (0.61 MB PDF)

Figure S2 List of contents contained in fraction #5 and #6
Found at: doi:10.1371/journal.pone.0013280.s002 (0.68 MB EPS)

Figure S3 Cell toxicities of Yokukansan and its components. Cell toxicity of N2a cells was measured based on morphological changes. Cell death was measured 24 h after treatment with Yokukansan or each of the indicated components of Yokukansan (200 μg/ml). Non treated cells were used as a control. Quantitative data are expressed as the mean ± SEM for at least three independent experiments. The P value was compared with the control and calculated by Student's T test.
Found at: doi:10.1371/journal.pone.0013280.s003 (0.68 MB EPS)

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Author Contributions

Conceived and designed the experiments: TH S. Matsuzaki TK MT. Performed the experiments: TH S. Matsuzaki MK. Analyzed the data: TH S. Matsuzaki S. Miyata KK SN TK MT. Wrote the paper: TH S. Matsuzaki.

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技術

キメラGタンパク質を用いたGタンパク質共役型受容体（GPCR）のアッセイ

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実験のコツと注意点

本稿ではGタンパク質共役型受容体（GPCR：G protein-coupled receptor）とGタンパク質のサブタイプの適切なマッチング（簡単にいうと相性）が機能発現に重要であることについては解説しているが，GPCRの発現実験の際にその他注意すべき点として，GPCRと発現させる細胞の種類との相性も重要である．組み合わせによっては，GPCRが細胞膜に十分に移行されない場合がある．このような場合の対処法として，ロドプシンのN末端のアミノ酸配列を目的のGPCRに付け加えることにより，細胞膜への移行性が向上し，問題点が解決する場合がある．

はじめに

Gタンパク質共役型受容体（GPCR：G protein-coupled receptor）は，細胞膜を7回貫通する受容体で，ヒトの遺伝子ファミリーの中でも最も大きく，タンパク質をコードしている遺伝子全体の3～4%に相当し，ホルモン受容体，神経伝達物質受容体，感覚器の受容体（視覚，嗅覚，味覚）など幅広い生理機能に関与する．この遺伝子ファミリーは，多くの疾患とも密接に関係し，現在使用されている全ての薬の約30%が，このGPCRを標的分子としたものである．このようにGPCRに関しての多くのことが解明されてきたにもかかわらず未だに機能の分かっていないorphan receptorが100以上存在している．これらの機能を解明していくことは，未だ分かっていない生理機能の解明，病態の解明，薬剤の開発に繋がるものである．

GPCRはαβγの三量体のGタンパク質とカップリングすることによって，リガンドと結合した情報を，エフェクター，セカンドメッセンジャーに伝える．GPCRと直接結合するGαは大きく分類するとGq，Gs，Giの3種類が存在する．Gqはフォスフォライペースcを活性化し，ジアシルグリセロールとIP_3を産生し，小胞体上のIP_3受容体を介して細胞内のカルシウムイオン濃度を上昇させる．一方，Gsはアデニレートサイクレースを活性化し細胞内のcAMPを増加させ，Giはアデニレートサイクレースを抑制し細胞内のcAMPを減少させる．このようにGPCRのシグナルは異なるセカンドメッセンジャー系に出力されるので，その機能を解析するために細胞内カルシウムイメージング法，cAMPアッセイ，GTPγSアッセイなどさまざまな手法を選択しなければならない[1,2)]．例えばセロトニン受容体では5-HT1受容体はGi，5-HT2受容体はGq，5-HT4受容体はGs，5-HT5受容体はGi，5-HT6～7受容体はGsのGαタンパク質と共役するため，一つの薬剤のセロトニン受容体に対する特異性を調べるためには，異なる条件やアッセイ法を用

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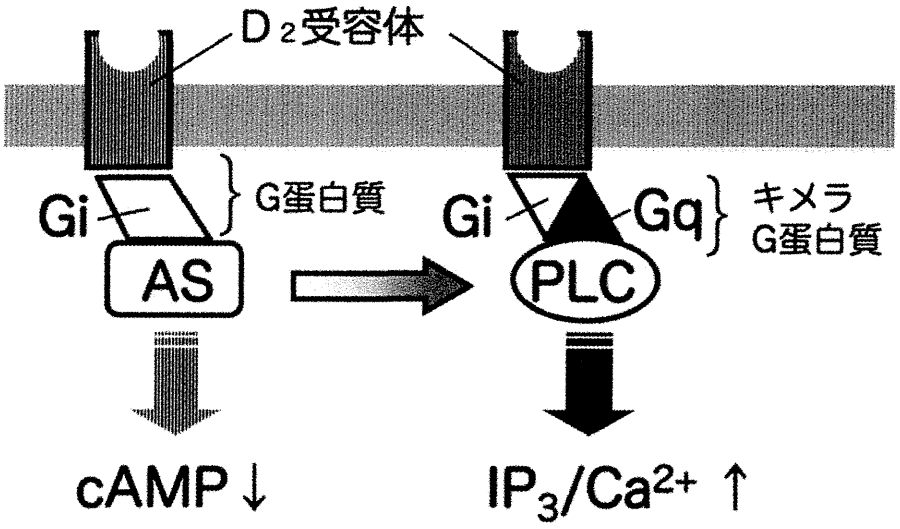

図1　キメラGタンパク質によるドーパミンD2受容体のアッセイ
ドーパミンD_2受容体はGiタンパク質と共役しているため，ドーパミンの刺激はアデニレートサイクレースを抑制してcAMPを減少させる．一方，GiとGqのキメラGタンパク質の存在下ではドーパミンのシグナルが，フォスフォライペースCを活性化させる信号に変換され，最終的に細胞内のカルシウムイオン濃度を上昇させる．AS：アデニレートサイクレース，PLC：フォスフォライペースC

いて各種セロトニン受容体サブタイプに対する親和性を比較しなければならはい．また，orphan receptorのリガンドを検索する際にもアッセイ法によっては活性を見落としてしまう場合がある．

本稿では，このような問題点を解消するため，各種GPCRのシグナルをカルシウムイメージング法により同一プラットホーム上でアッセイできるように，キメラGタンパク質を用いた解析法について解説する．**図1**で簡単に説明すると，Gαタンパク質のN末端側はエフェクターと結合する部分で，C末端側はGPCRと結合する部分なので，N末端側をGqのタンパク質，C末端側をGiのタンパク質に置換したキメラGタンパク質を用いると，例えばドーパミンD_2受容体のようなGiと共役するGPCRのシグナルをcAMPの減少ではなく，細胞内カルシウム濃度の上昇として出力し，カルシウムイメージング法で解析できるようになる．

I. Gタンパク質におけるGPCRとの結合に必要な構造

キメラGαタンパク質を作成する際に，Gαタンパク質のC末端側のどの領域がGPCRと結合するために必要不可欠かを検討するために以下の実験を行った[3]．GPCRとしては味覚受容体であるT2R5とT2R16を用いた．キメラGαタンパク質の作成にあたっては，細胞内のカルシウムイメージング法でアッセイするためにN末端側はGq系のG16を用い，T2R5やT2R16と共役させるためC末端側はGi系のgustducin（Ggust）を用いた．**図2A**のようにキメラGタンパク質（G16/gust）においてgustducin由来のC末端のアミノ酸残基を5，11，23，37，44の順で増加させていくと，37，44でGPCRのT2R5とT2R16を認識し，機能発現ができるようになった．つまり，Gαタンパク質のC末端領域におけるβ6 sheet，α5 helix，extreme C terminusの構造が，Gタンパク質とGPCRとの機能的な結合に必要不可欠であるということが分かった．また，**図2B**で，これらのキメラGαタンパク質が実際に発現していることをイムノブロットで確認した[3]．

図3は図2で行ったT2R5，T2R16のカルシウムイメージング法による実際のアッセイを示す．キメラG16/gustタンパク質を用いて，T2R5のリガンドであるcycloheximideを投与すると細胞内のカルシウム

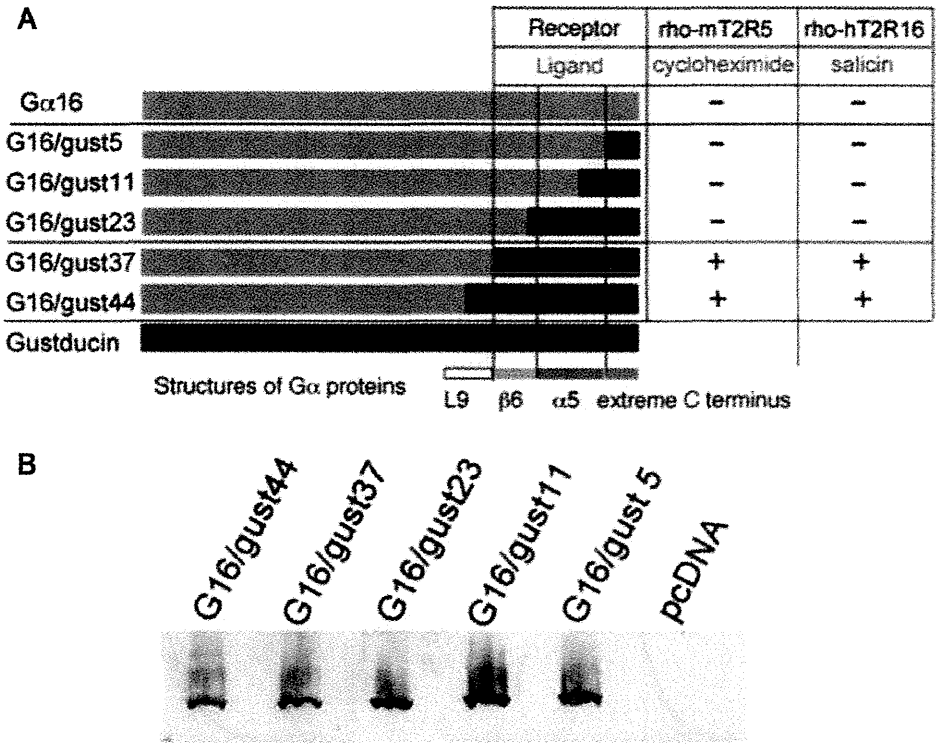

図2　Gタンパク質のC末端におけるGPCRの認識部位の同定
A：gustducin由来のC末端のアミノ酸残基を5，11，23，37，44の順で置換したキメラGタンパク質（G16/gust）を用いてGPCRのT2R5とT2R16のアッセイを行った．G16/gust37とG16/gust44において機能が発現した．B：これらのキメラGαタンパク質が実際に発現していることをイムノブロットで確認した．

濃度が上昇した（**図3A**）．T2R5とT2R16のそれぞれのリガンドであるcycloheximideとsalicinに対する応答は，濃度依存性であった（**図3B**）[3]．

II. キメラGタンパク質によるドーパミンD2L受容体の機能発現

ドーパミンD2L受容体の機能を解析するため，3種類のGi遺伝子ファミリー（Go，Gi2，Gi3）とG16との間でG16/o，G16/i2，G16/i3のキメラGタンパク質を作成し，HEK293T細胞にD2L受容体とそれぞれのキメラGタンパク質を共発現させてカルシウムイメージング法によりアッセイを行った[4]．G16/o，G16/i2，G16/i3を用いたアッセイ系ではドーパミンに対してそれぞれ高い親和性（EC50が3.2nM（G16/i3），10.4nM（G16/i2））を示しているのに対して，G15やG16のみを用いた系では親和性が著しく低下した（**図4A**）[4]．G16/o，G16/i2，G16/i3のキメラGタンパク質の存在下で，ドーパミン，D2アゴニストのキンピロール，D2パーシャルアゴニストのS-(-)-3PPPのD2L受容体に対する親和性を解析したところ，以前に報告されているcAMPアッセイやMAPKアッセイで得られた結果とほぼ同様の親和性が得られた（**図4B, C, D**）[5, 6]．また，S-(-)-3PPPはキメラGタンパク質を用いた実験においてもパーシャルアゴニストとしての特徴を示した．つまり，ドーパミンD2L受容体の機能解析に関して，キメラG16/iタンパク質を用いたカルシウムイメージングアッセイ系では，内因性のリガンド，アゴニスト，パーシャルアゴニストに対して以前から報告されている他のアッセイ系での結果と類似した結果が得られたことから，このキメラGタンパク質を用いた解析法は有用であると考えられた．

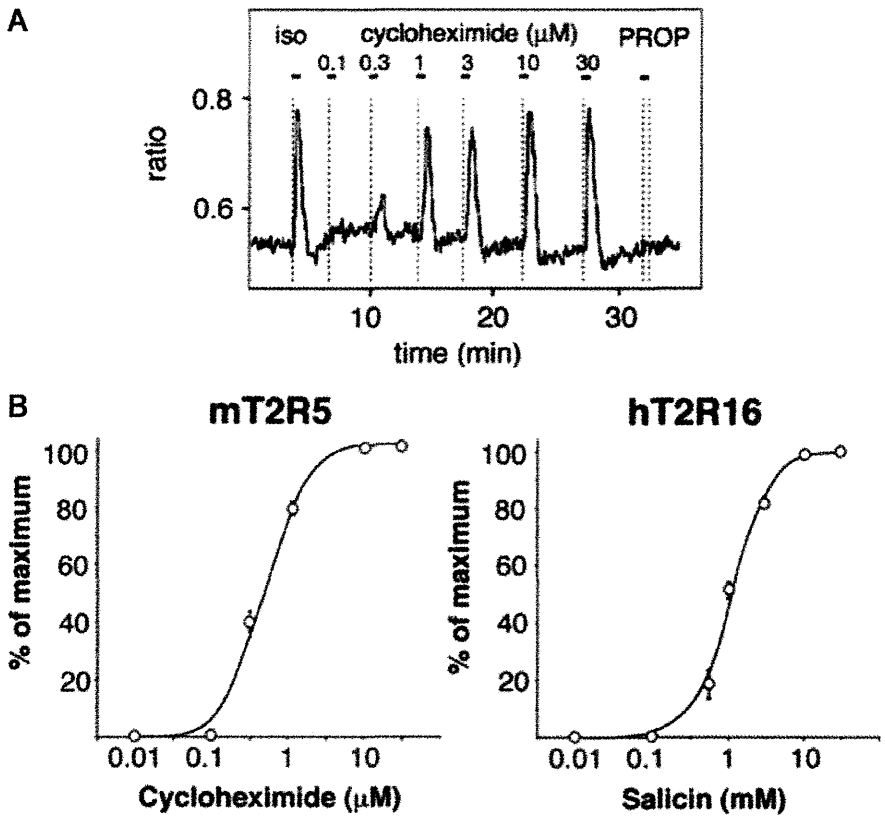

図3　G16/gustキメラタンパク質を用いたT2R5，T2R16のアッセイ
A：T2R5のcycloheximide投与に対する細胞内のカルシウム濃度の上昇．
B：T2R5とT2R16のそれぞれのリガンドであるcycloheximideとsalicinに対する応答の濃度依存曲線．

III. キメラGタンパク質による5-HT1A受容体の解析

D2L受容体と同様に3種類のGi遺伝子ファミリー（Go，Gi2，Gi3）とG16との間で作成したG16/o, G16/i2，G16/i3のキメラGタンパク質を用いて，5-HT1A受容体の機能を解析した[4]．**図5A**で示すようにG16とGiの3種類のキメラタンパク質を用いた実験では，D2L受容体の場合とは異なり，セロトニンの5-HT1A受容体に対する親和性は共役するGiタンパク質の種類により大きく異なった．G16/i3を用いた場合，EC50は2.6 nMで，G16/i2を用いた場合のEC50は46.5 nMであった[4]．このように共役するGタンパク質の種類によってセロトニンの5-HT1A受容体に対する親和性が異なってくる特性は以前にも報告されており[7]，今回の結果からキメラGタンパク質においてもこの特徴を保持していることが分かった[8,9]．一方，5-HT1A受容体はG15やG16を単独で発現させた場合にもそれらのGタンパク質と共役し機能を発現したが，セロトニンに対する親和性はG16/i3に比べると100倍程度低いものであった．

5-HT1A受容体のアンタゴニストであるスピペロンの阻害作用を5-HT1A受容体とG16/oを共発現させたHEK293T細胞を用いて解析したところ（**図5B**），スピペロンはセロトニンの5-HT1A受容体に対する応答を濃度依存的に抑制した（IC50は560 nM）[4]．この結果や前の実験結果から総合的に判断すると，適切なキメラGタンパク質を用いれば，このキメラGタ

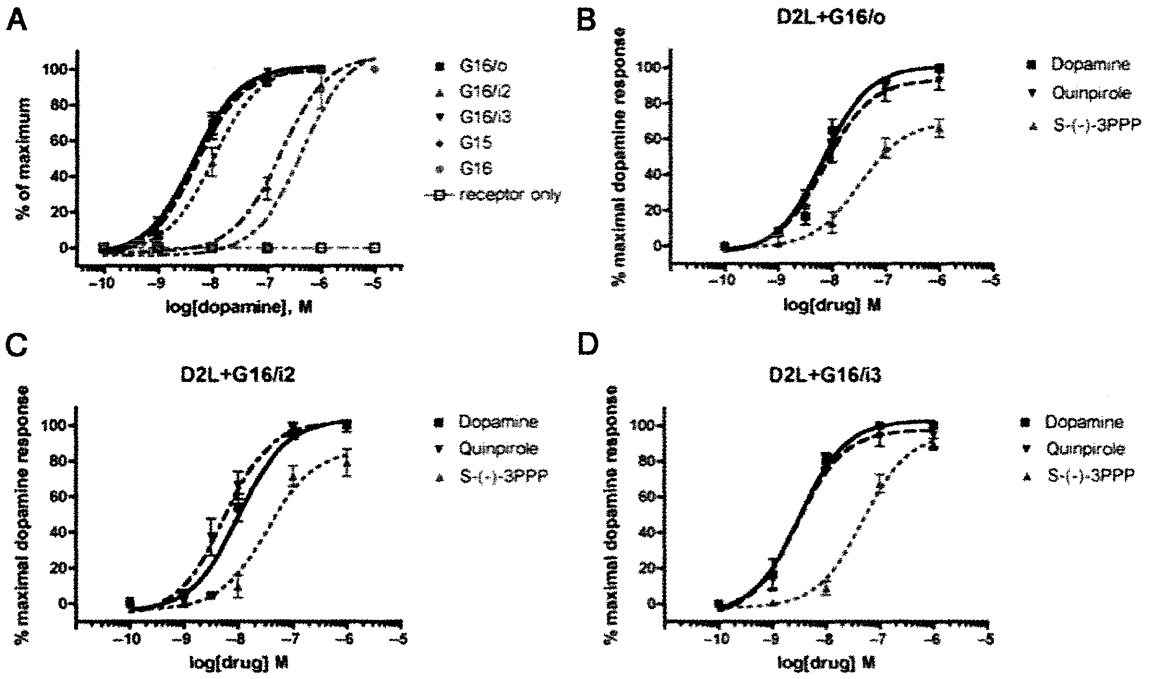

図4　キメラGタンパク質によるドーパミンD2L受容体の発現

ドーパミンD2L受容体とG16/o，G16/i2，G16/i3のキメラGタンパク質をHEK293T細胞に共発現させて，D2L受容体の薬理学的解析を行った．A：D2L受容体に対するドーパミンの親和性及びキメラGタンパク質の影響．B，C，D：G16/o，G16/i2，G16/i3のキメラGタンパク質の存在下におけるドーパミン，D2アゴニストのキンピロール，D2パーシャルアゴニストのS-(-)-3PPPのD2L受容体に対する濃度応答曲線．

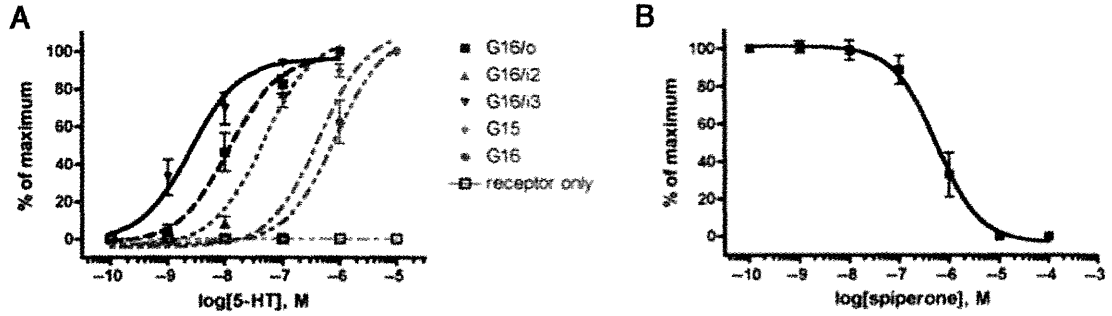

図5　キメラGタンパク質による5-HT1A受容体の解析

G16/o，G16/i2，G16/i3のキメラGタンパク質を用いて5-HT1A受容体の機能を解析した．A：セロトニンの5-HT1A受容体に対する濃度依存曲線を示す．セロトニンの5-HT1A受容体に対する親和性は共役するGiタンパク質の種類により大きく異なった．B：5-HT1A受容体とG16/oを共発現させたHEK293T細胞を用いて5-HT1A受容体のアンタゴニストのスピペロンの阻害作用を解析した．

ンパク質／カルシウムイメージング法では，アゴニスト，アンタゴニスト，パーシャルアゴニストなどの薬剤とGPCRとの相互関係は保持されると考えられた．

おわりに

本稿では，主にGiと共役するGPCRに関してキメラGタンパク質を用いてカルシウムイメージングでアッセイする方法について解説した．一方，Gsと共役するGPCRに関しても，G16/sのキメラGタンパク質を作成すれば，同様にカルシウムイメージング法によって機能解析できるが，この場合はもっと簡単にG15やG16を直接GPCRと共発現してもカルシウムイメージング法で解析できる[10]．このように，G16/i，G16を用いることにより，Gi，Gs，Gqと共役する全てのGPCRをカルシウムイメージング法という共通のプラットホーム上で解析ができるようになり，このテクニックはハイスループットスクリーニングなどの技術への応用に適していると考えられる．

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