## NEUROSCIENCE

# HSP105 prevents depression-like behavior by increasing hippocampal brain-derived neurotrophic factor levels in mice

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Heat shock proteins (HSPs) are stress-induced chaperones that are involved in neurological disease. Although increasingly implicated in behavioral disorders, the mechanisms of HSP action, and the relevant functional pathways, are still unclear. We examined whether oral administration of geranylgeranylacetone (GGA), a known HSP inducer, produced an antidepressant effect in a social defeat stress model of depression in mice. We also investigated the possible molecular mechanisms involved, particularly focusing on hippocampal neurogenesis and neurotrophic factor expression. In stressed mice, hippocampal HSP105 expression decreased. However, administration of GGA increased HSP105 expression and improved depression-like behavior, induced hippocampal cell proliferation, and elevated brain-derived neurotrophic factor (BDNF) levels in mouse hippocampus. Co-treatment with GGA and the BDNF receptor inhibitor K252a suppressed the antidepressant effects of GGA. HSP105 knockdown decreased *BDNF* mRNA levels in HT22 hippocampal cell lines and hippocampal tissue and inhibited the GGA-mediated antidepressant effect. These observations suggest that GGA administration is a therapeutic candidate for depressive diseases by increasing hippocampal BDNF levels via HSP105 expression.

## INTRODUCTION

Heat shock proteins (HSPs), which act as molecular chaperones, play an important regulatory role for the maintenance of cellular homeostasis (*1*, *2*). HSPs are subdivided into distinct families based on their molecular weight and sequence homology: HSP110, HSP90, HSP70, HSP60, HSP40, and low–molecular weight HSPs. Each HSP family has specific functions that have been the subject of different reviews (*3*, *4*). There is increasing evidence that HSPs are associated with neuronal protection. HSP70 and HSP40 abrogate polyglutamine toxicity by refolding and solubilizing pathogenic proteins (*5–7*). HSP70 also regulates the activity of glucocorticoid receptors, which play an important role in depression (*8*). Localization studies have shown a wide distribution of HSP110 in the cerebral cortex, hippocampus, thalamus, and hypothalamus (*9*). HSP90 is preferentially expressed in the nonstressed mammalian brain (*10*). In environmentally stressed mice, the expression of HSP90, HSP70, and HSP105 (a member of HSP110) in the hippocampus is altered (*11*). Thus, a huge body of evidence has accumulated, suggesting that the activities of HSPs are associated with various behavioral disorders. These reports allow us to hypothesize that HSPs are a new therapeutic target for psychological disorders, including depression.

Geranylgeranylacetone (GGA) is an acyclic isoprenoid compound, which has been widely used as an anti-ulcer agent. GGA has been reported to induce HSP synthesis in various tissues, including the gastric mucosa, liver, and central nervous system (*12–14*). Oral administration of GGA induces HSP70 expression in the rat brain and plays an important role in ischemic brain injury (*14*, *15*). However, it is still unclear whether GGA is associated with the improvement of depression-like behavior.

The psychopathology of depression is multifaceted and is known to be accompanied by a decrease or impairment in neurogenesis in the hippocampus (*16*, *17*). The relationship between HSPs and hippocampal neurogenesis has been reported. HSPB8 has been shown to be neuroprotective in the mouse adult hippocampus (*18*). Furthermore, HSP27 (HSP25 is the mouse homolog of the human HSP27) is neuroprotective against the harmful effects of ethanol administration in the mouse brain (*19*). However, the effect of GGA administration on hippocampal neurogenesis in mice remains unclear. Here, we examined whether oral GGA administration induced an antidepressant effect in a social defeat stress model of depression in mice. We also investigated the possible molecular mechanisms involved, particularly focusing on hippocampal neurogenesis and neurotrophic factor expression. Furthermore, we evaluated the association of teprenone (pharmaceutical name of GGA) use and drug-induced depression using the U.S. Food and Drug Administration (FDA) Adverse Event Reporting System (FAERS), a large and useful database of adverse events.

## RESULTS

### Effects of social defeat stress on HSP mRNA expression in the mouse hippocampus

To examine the effects of social defeat stress on HSPs, we used quantitative reverse transcription polymerase chain reaction (PCR) to detect *HSP25*, *HSP60*, *HSP72*, and *HSP105* mRNA level changes in the hippocampus after stress exposure. To induce social defeat stress, we subjected mice daily to 10-min defeat sessions, followed by continuous protected sensory contact with their aggressor for a total of 15 days. Each day, experimental mice were exposed to a new resident home cage. *HSP25*, *HSP60*, and *HSP72* mRNA levels did not show any significant differences between control and stressed mice. However, *HSP105* mRNA levels were significantly decreased when compared to control ($t$ test, $P = 0.0061$; Fig. 1).

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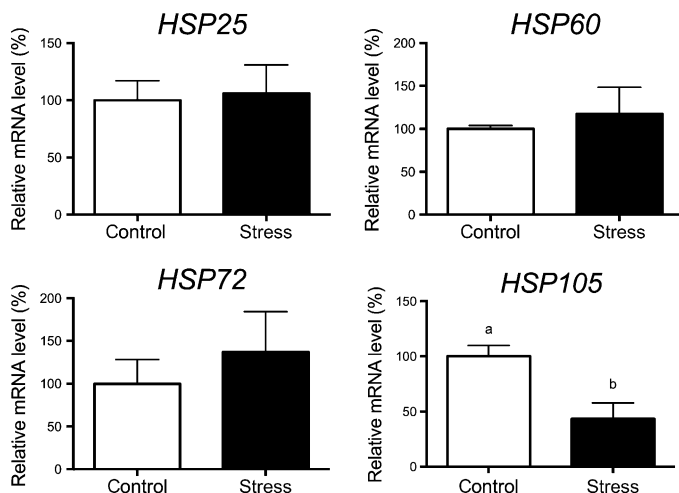*Corresponding author. Email: hobara@dls.ous.ac.jp

**Fig. 1. Stress-induced *HSP* mRNA level changes in the hippocampus.** Quantitative reverse transcription PCR analysis showing the expression levels of *HSP25*, *HSP60*, *HSP72*, and *HSP105* ($n$ = 4 to 5 animals per group). Each bar indicates the mean ± SEM. Statistically different groups are indicated by letters.

## Oral administration of GGA induces HSP expression in the mouse hippocampus and rescues depression-like behavior by increasing hippocampal cell maturation

Because we found decreased *HSP105* mRNA expression in the hippocampus following stress exposure, we next determined whether HSP105 activity plays a role in behavioral development. To demonstrate whether or not GGA could increase hippocampal HSPs, 8-week-old mice received GGA [0.34% (w/v)] in their drinking water for 15 days. Following GGA administration, we found significantly increased *HSP25* and *HSP60* levels compared with vehicle-treated mice (Fig. 2, A and B). Further, *HSP72* levels were increased in stressed mice and after GGA treatment (Fig. 2C). In contrast, *HSP105* levels were reduced in stressed mice but increased by GGA (Fig. 2D). In addition, we measured HSP105 protein expression levels. GGA and stress resulted in a significant interaction, with stress-reducing HSP105 protein in stressed mice only (Fig. 2E).

To investigate the effects of exogenously increased HSPs on depression-like behavior in stressed mice, we monitored social interaction time. Stressed mice spent less time interacting with the unfamiliar CD1 mouse (Fig. 3A). This social avoidance is considered a measure of depression-like behavior because it can be reversed by chronic, but not acute, treatment with antidepressants (20). GGA treatment improved defeated mouse interaction time (Fig. 3A). We also examined depression-like behavior using a forced swim test, tail suspension test, and sucrose preference test. We found significantly increased immobility time in the forced swim test after stress, which was decreased by GGA (Fig. 3B). Similarly, immobility time in the tail suspension test was significantly increased in stressed mice (Fig. 3C). Sucrose preference displayed the same social interaction pattern, with stress decreasing and GGA increasing sucrose preference (Fig. 3D). In contrast, total fluid consumption was increased by stress (Fig. 3E). These results suggest that GGA treatment induces antidepressant-like effects in stressed mice. In addition, we also examined the general behavior of mice in the open-field test. There were no differences in locomotor activity, rearing activity, or time spent in the center area of the open-field test (Fig. 3F).

Next, we analyzed granule neuron development using doublecortin (DCX). Stressed mice had fewer DCX-positive cells in the subgranular

zone of the dentate gyrus compared with nonstressed mice (Fig. 3G). The number of DCX-positive cells in the granule cell layer of the dentate gyrus was significantly decreased in stressed mice. GGA administration reduced the number of stress-induced DCX-positive cells. In addition, we determined whether mature neuronal development is altered in mouse brain sections immunostained with anti–neuronal nuclei (NeuN), a widely used neuronal marker. However, we found no differences in NeuN cells following stress or GGA treatment (fig. S1). Thus, the social defeat stress model showed reduced neural progenitor proliferation in the dentate gyrus, and GGA administration rescued dentate gyrus maturation.

## GGA-mediated HSP expression increases BDNF levels in the hippocampus

We next determined if GGA-induced HSP expression improved depression-like behavior by increasing hippocampal neural progenitor proliferation. For this, we focused on neurotrophic factors in the mouse hippocampus. According to previous reports (21–24), the role of neurotrophins, including brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), neurotrophin 3 (NT-3), and nerve growth factor (NGF), in depression-like behavior has been well studied. In the stressed with GGA treatment group, *BDNF*, but not *NGF*, *CNTF*, or *NT-3*, mRNA expression was significantly higher (Fig. 4, A to D). We also investigated BDNF protein expression by Western blotting and immunohistochemistry and found a similar pattern as for *BDNF* mRNA, with GGA increasing BDNF protein levels (Fig. 4E and fig. S2).

## The BDNF receptor inhibitor K252a suppresses GGA-induced antidepressant-like effects

To further characterize the involvement of BDNF expression on the GGA-mediated antidepressant effect, we inhibited BDNF activation using a BDNF receptor inhibitor (K252a; 25 µg/kg per day) during stress exposure in mice. Following GGA administration, the social interaction rate significantly increased (Fig. 5A). In contrast, immobility time was not significant in either the forced swim test or tail suspension test, but K252a inhibited the GGA-dependent antidepressant effect (Fig. 5B, $P$ = 0.059; Fig. 5C, $P$ = 0.055). Further, sucrose preference was elevated in stressed mice by GGA, but reduced by K252a, with total intake unaffected (Fig. 5D). In contrast, K252a treatment did not significantly affect general motor behavior in the open-field test (locomotor activity, rearing activity, and time spent in the center area) (Fig. 5E). We also determined whether combined K252a and GGA influences nonstress mouse behavior. However, only total fluid intake was significantly reduced by GGA (fig. S3).

## HSP105-siRNA treatment suppresses the expression of BDNF

We have already shown that social defeat stress significantly decreases HSP105 but not other HSPs. GGA administration increased HSPs and BDNF and abolished stress-induced depression-like behavior. From these results, we hypothesized that HSP105 plays a critical role in the GGA-mediated antidepressant effect via BDNF elevation. To elucidate the effects of HSP105 knockdown on BDNF expression in hippocampus cells, we administered either HSP105–small interfering RNA (siRNA) or nontargeting control siRNA in HT22 cells and exposed them to 50 µM GGA at 41°C for 2 hours. A marked reduction in *HSP105* mRNA was observed in HT22 cells (one-way ANOVA, $P$ < 0.05; Fig. 6A). At the same time, the siRNA decreased the level of *BDNF* mRNA (one-way ANOVA, $P$ < 0.05; Fig. 6B). Next, to more
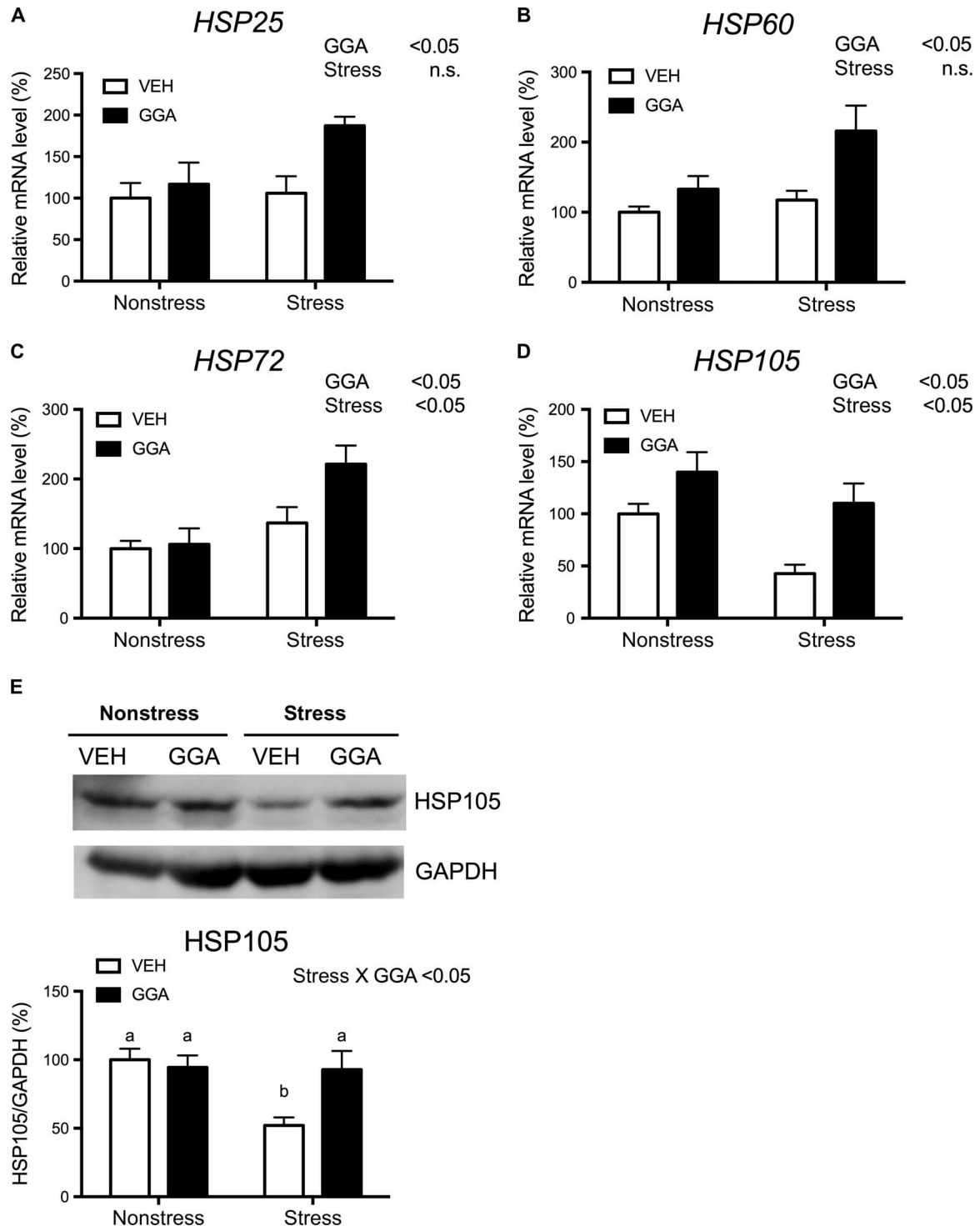
**Fig. 2. Effects of GGA on HSP expression in the hippocampus.** The level of *HSP* mRNA in the hippocampus was detected by quantitative reverse transcription PCR. *HSP25* mRNA (**A**), *HSP60* mRNA (**B**), *HSP72* mRNA (**C**), *HSP105* mRNA (**D**), and HSP105 protein (**E**) ($n = 5$ to 6 animals per group). Each bar indicates the mean ± SEM, with significant differences as inserts on the chart ($P < 0.05$). Groups were first tested by two-way analysis of variance (ANOVA). Then, if a statistical interaction was observed between factors, comparison of all four groups was performed by one-way ANOVA with Tukey's post hoc test. Statistically different groups are indicated by letters. n.s., not significant; VEH, vehicle; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
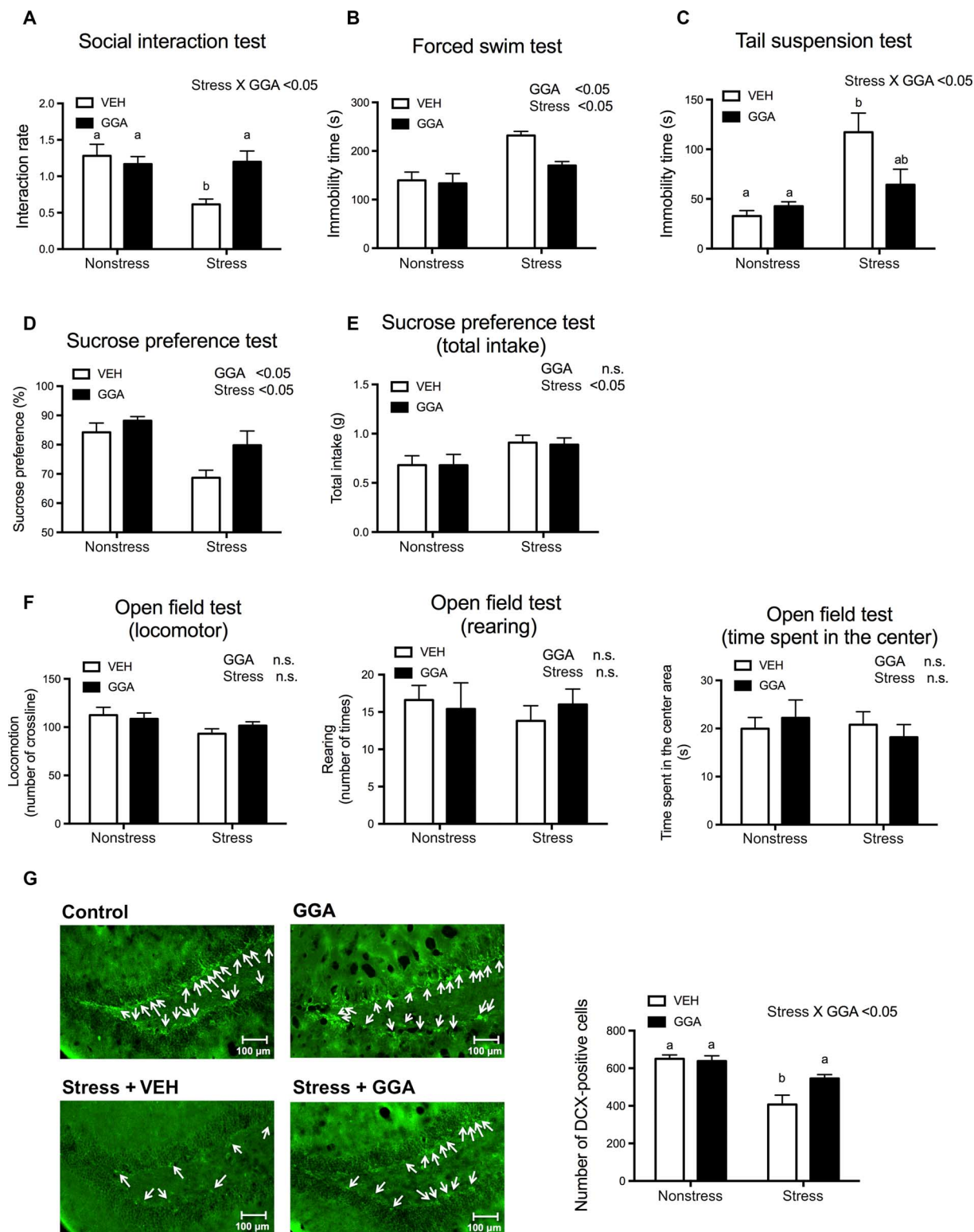
**Fig. 3. GGA administration rescues depression-like behavior and hippocampal proliferation.** (**A**) Interaction rate in the interaction test. (**B**) Immobility times in the forced swim test. (**C**) Immobility times in the tail suspension test. Sucrose preferences (**D**) and total fluid intake (**E**) in the sucrose preference test. (**F**) General behavior in the open-field test locomotor activity, rearing activity, or time spent in the center area ($n = 4$ to 5 animals per group). (**G**) Photomicrographs of the dentate gyrus showing representative DCX-positive cells. Scale bar, 100 μm. Arrows show immunopositive cells. Right: Number of DCX-positive cells. Each bar indicates the mean ± SEM, with significant differences shown as inserts on the chart ($P < 0.05$). Groups were first tested by two-way ANOVA. Then, if a statistical interaction was observed between factors, comparison of all four groups was performed by one-way ANOVA with Tukey's post hoc test. Statistically different groups are indicated by letters.
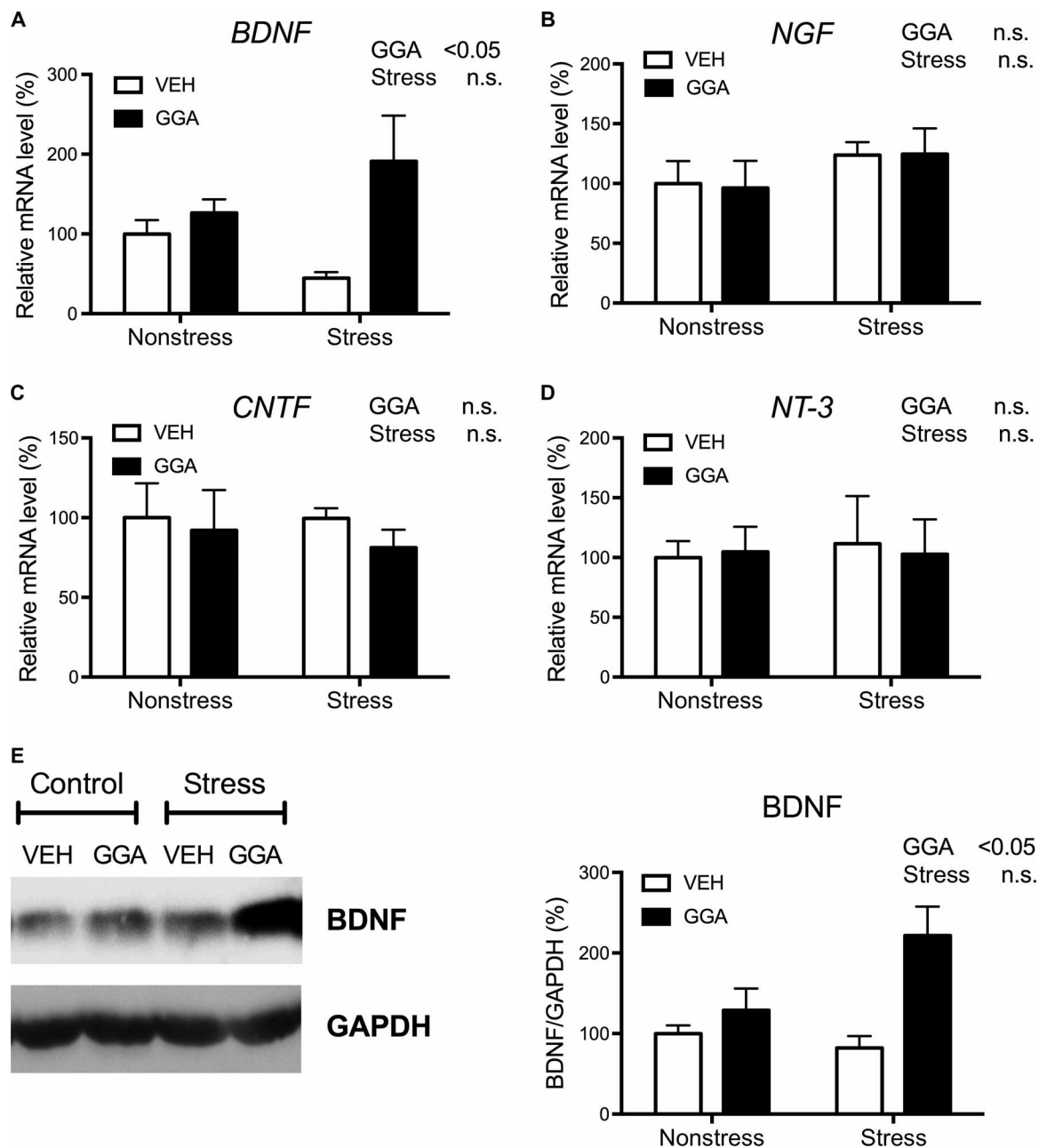
**Fig. 4. Effects of GGA administration on mRNA levels of neurotrophic factors in the stressed mouse hippocampus.** Mice were administered vehicle or GGA orally. Quantitative reverse transcription PCR analysis showing the expression levels of (**A**) *BDNF*, (**B**) *NGF*, (**C**) *CNTF*, and (**D**) *NT-3* ($n = 5$ animals per group). (**E**) Western blotting of BDNF protein expression in mouse hippocampus ($n = 4$ to 5 animals per group). Each bar indicates the mean ± SEM, with significant differences shown as inserts on the chart ($P < 0.05$). Groups were first tested by two-way ANOVA. Then, if a statistical interaction was observed between factors, comparison of all four groups was performed by one-way ANOVA with Tukey's post hoc test. Statistically different groups are indicated by letters.

specifically analyze the role of HSP105 on BDNF expression in vivo, we injected HSP105-siRNA or nontargeting control into the mouse brain, and after 24 hours, HSP105 protein expression was evaluated by Western blotting. HSP105-siRNA significantly decreased HSP105 levels by ~80% in the mouse hippocampus compared with the nontargeting control (Fig. 6C). We also investigated HSP25, HSP60, and HSP72 expression in HSP105-siRNA mouse hippocampus but found no significant changes in HSP expression (fig. S4). Next, we injected HSP105-siRNA

into the mouse brain on days 1, 7, and 14 during a 15-day stress exposure. We observed that the decreases seen in immobility time in the forced swim test and tail suspension test following GGA treatment in stressed mice were absent in HSP105-siRNA knockdown mice (one-way ANOVA, $P < 0.05$; Fig. 6, D and E). Furthermore, the increase in social interaction rate induced by GGA administration was absent in HSP105-siRNA (one-way ANOVA, $P < 0.05$; Fig. 6F). In the sucrose preference test, HSP105-siRNA did not further affect sucrose preference
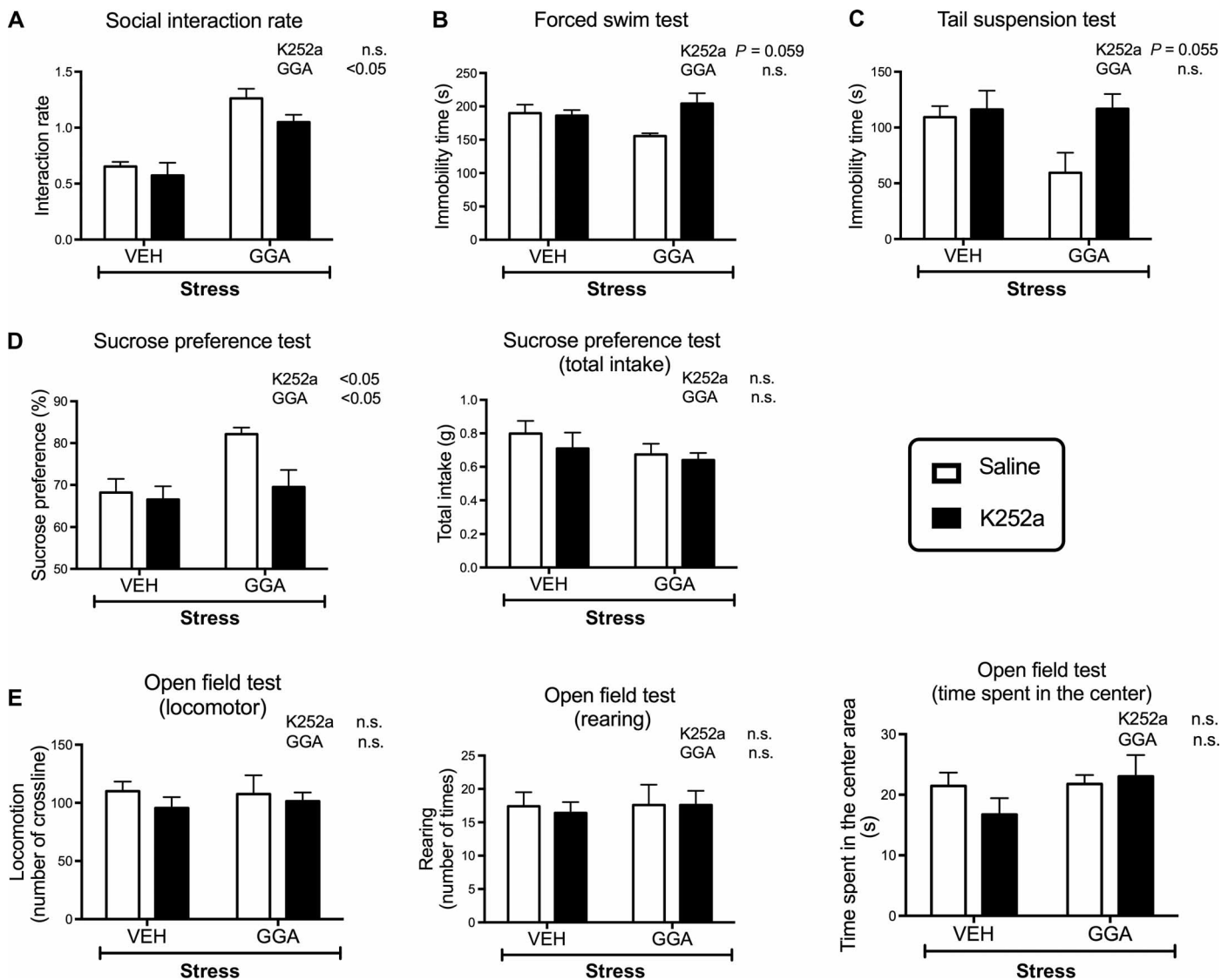
**Fig. 5. The BDNF receptor inhibitor K252a suppresses GGA-induced antidepressant-like effects.** (**A**) Social interaction rate in the interaction test. (**B**) Immobility times in the forced swim test. (**C**) Immobility times in the tail suspension test. (**D**) Sucrose preference and total fluid intake in the sucrose preference test. (**E**) No significant effect on general behavior, locomotor activity, rearing activity, or time spent in the center area in the open-field test ($n = 5$ animals per group). Each bar indicates the mean ± SEM, with significant differences shown as inserts on the chart ($P < 0.05$). Groups were first tested by two-way ANOVA. Then, if a statistical interaction was observed between factors, comparison of all four groups was performed by one-way ANOVA with Tukey's post hoc test. Statistically different groups are indicated by letters.

and total fluid intake (Fig. 6G). Combining HSP105-siRNA and GGA treatment significantly decreased BDNF levels in stressed mouse hippocampus (one-way ANOVA, $P < 0.05$; Fig. 6H).

**FAERS database**

The characteristics of the study population are presented in Table 1. The number of cases, which claimed depressive events (major depression, agitated depression, or depression suicidal) during the study period, was 49,839. Among those, 15,353 reports were male and 34,486 reports were female. The mean age of cases was 48.3 years. In contrast, the number of non-cases, which include events other than depression, was 2,277,014. Among those, 856,772 reports were male and 1,420,242 reports were female. The mean age of non-cases was 54.1 years. The data on teprenone (GGA)–associated depression are presented in Table 2. It is well known that administration of peginterferon α-2a, peginterfer-

on α-2b, or ribavirin elicits depressive events (25, 26). Because of these previous reports, we focused on drugs that cause drug-induced depression. The number of reports, where subjects were administered either peginterferon α-2a, peginterferon α-2b, or ribavirin, but not teprenone (GGA), and claimed depressive events was 1626 (males: 904; females: 722). Coadministration of teprenone (GGA) decreased the number of reports, with the number of subjects administered peginterferon α-2a, peginterferon α-2b, or ribavirin and claiming depressive events being 10 (males, 5; females, 5). In contrast, the number of subjects administered peginterferon α-2a, peginterferon α-2b, or ribavirin, but not teprenone (GGA), and claiming other events unrelated to depression was 41,348 (males, 23,393; females, 17,955). Coadministration of teprenone (GGA) decreased the number of reports to 403 (males, 196; females, 207). If no teprenone (GGA) was used, the reporting odds ratio (ROR) for the use of peginterferon α-2a, peginterferon α-2b, or ribavirin was
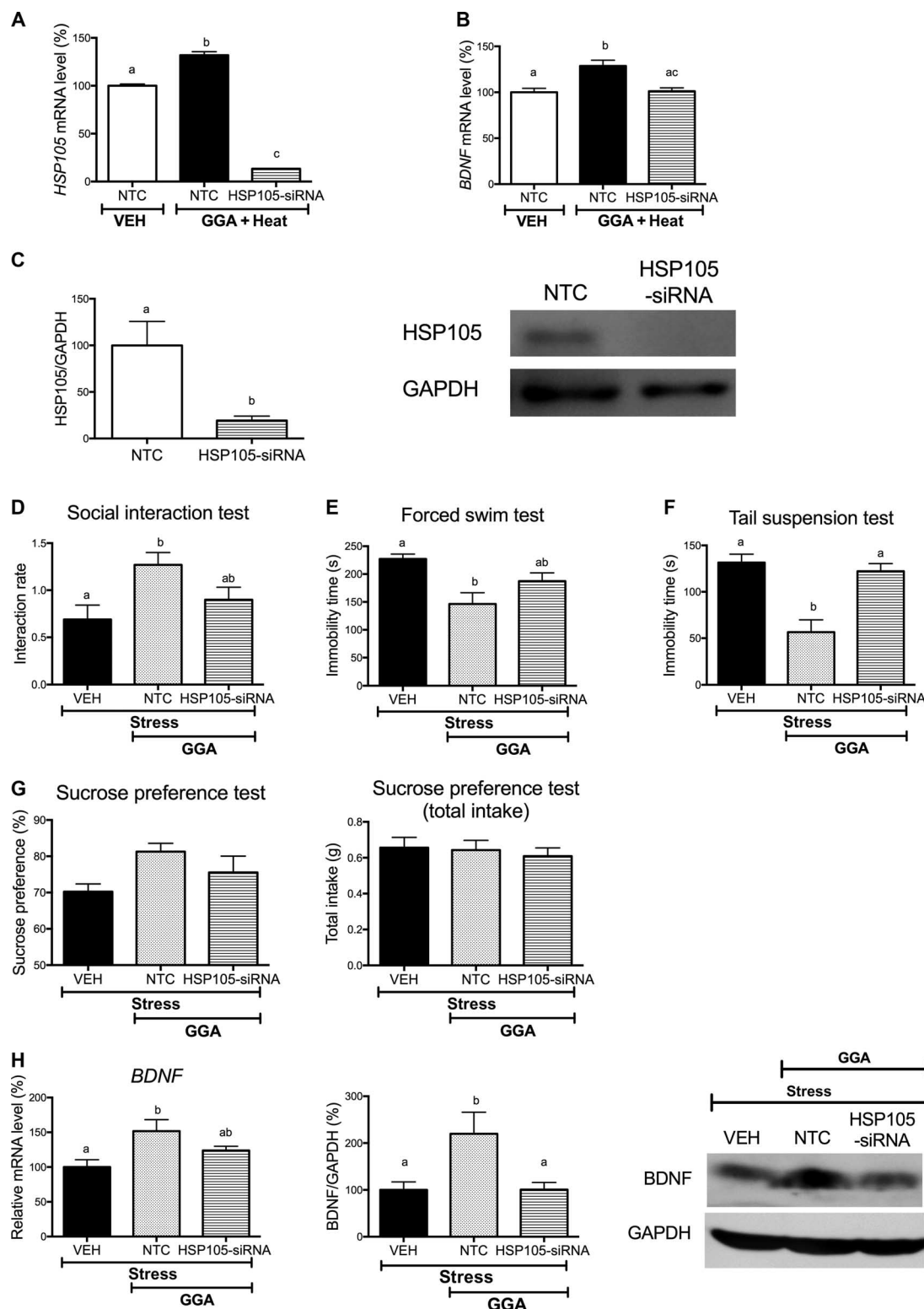
**Fig. 6. HSP105-siRNA in HT22 cells or stressed mouse brain decreased BDNF levels and abolished antidepressant effects.** (**A**) Level of *HSP105* mRNA in HT22 cells (ANOVA, $F_{2,13} = 544.1$, $n = 5$ to 6). (**B**) Level of *BDNF* mRNA in HT22 cells (ANOVA, $F_{2,13} = 10.74$, $n = 5$ to 6). (**C**) The level of HSP105 protein in the hippocampus was detected by Western blotting ($n = 3$). (**D**) Immobility times in the forced swim test (ANOVA, $F_{2,11} = 6.03$). (**E**) Immobility times in the tail suspension test (ANOVA, $F_{2,11} = 15.02$). (**F**) Social interaction rate in the interaction test (ANOVA, $F_{2,11} = 5.00$). (**G**) Sucrose preference and total fluid intake in the sucrose preference test. (**H**) Level of *BDNF* mRNA and protein in the mouse hippocampus (ANOVA, $F_{2,11} = 4.54$ and $F_{2,12} = 5.41$, respectively) ($n = 4$ to 5 animals per group). Each bar indicates the mean ± SEM. GGA + NTC, GGA with nontargeting control; GGA + HSP105-siRNA, GGA with HSP105-siRNA treatment. Statistically different groups are indicated by letters.

**Table 1. Characteristics of study population over 5 years (2010–2015) in the FAERS database.**

|  | Case with depression, n (%) | Non-cases, n (%) |
|---|---|---|
| Total | 49,839 (100) | 2,277,014 (100) |
| Male | 15,353 (30.8) | 856,772 (37.6) |
| Female | 34,486 (69.2) | 1,420,242 (62.4) |
| Mean age (years) | 48.3 | 54.1 |

**Table 2. Association of teprenone with depressive events in patient using peginterferon α-2a, peginterferon α-2b, or ribavirin. CI, confidence interval.**

|  | Cases with depression, n | Non-cases, n | Reporting odds ratio (95% CI) |
|---|---|---|---|
| Total (male and female) |  |  |  |
| Teprenone not present | 1626 | 41,348 | 1.82 (1.73–1.92) |
| Teprenone present | 10 | 403 | 1.13 (0.61–2.12) |
| Male |  |  |  |
| Teprenone not present | 904 | 23,393 | 2.23 (2.08–2.39) |
| Teprenone present | 5 | 196 | 1.42 (0.59–3.46) |
| Female |  |  |  |
| Teprenone not present | 722 | 17,955 | 1.67 (1.55–1.80) |
| Teprenone present | 5 | 207 | 0.99 (0.41–2.42) |

1.82 (95% CI, 1.73 to 1.91). If the users were administered teprenone (GGA), the ROR for peginterferon α-2a, peginterferon α-2b, or ribavirin was 1.13 (95% CI, 0.61 to 2.12), indicating that coadministration with teprenone (GGA) suppressed drug-induced depression by interferon.

## DISCUSSION

The present study shows that social defeat stress induces depression-like behavior by decreasing HSP105 in the mouse hippocampus. Moreover, we showed that the HSP inducer GGA can increase HSP105 expression, rescue mouse behavior, increase cell proliferation in the hippocampus, and increase levels of BDNF. Inhibition of the BDNF receptor abolished the GGA-mediated antidepressant effect. HSP105 knockdown also inhibited the GGA-mediated antidepressant effect. Furthermore, the FAERS database showed that combined treatment with GGA alleviated drug-induced depression in patients. Together, these results elucidate a novel function for GGA, suggesting that GGA-mediated HSP105 may enhance BDNF levels to alleviate depression-like behavior.

GGA is centrally acting after oral administration and has been used clinically for many years as a treatment for peptic ulcers without serious side effects (27). Yamagami et al. (13) have reported that GGA ad-

ministration augmented HSP72 expression in response to stress, but this effect was induced with some stress condition. However, we show here that GGA administration significantly increases HSPs and, in particular, *HSP105* mRNA in mouse hippocampus. It has been noted that HSP110 is highly expressed in the brain in a similar manner to that observed in heat-shocked cells (9). Furthermore, HSP105 expression in naïve mouse hippocampal neurons was higher than in damaged neurons (28). Thus, it is likely that HSP105 plays an important role in the brain and that HSP105 may be sensitive to external influences such as neuronal damage or GGA administration.

Although numerous reports have shown that GGA accelerates HSP70 expression and plays an important role in brain injury, HSP105 has not been thoroughly investigated (27, 29–31). HSP105 belongs to the HSP110 family. HSP110 is distributed in the cerebral cortex, hippocampus, thalamus, and hypothalamus (9). HSP105 inhibited stress-induced apoptosis in PC12 neuronal cells (32). Recent evidence demonstrated that chronic stress results in alterations to HSP105, with increased levels of caspase-3 in the female mouse hippocampus (11). Thus, HSP105 has been demonstrated to play a key role in neuroprotection. Consistent with this, the present study demonstrated that HSP105 decreases in stressed mice and elicits depressive-like behavior. Furthermore, GGA-mediated HSP105-induced BDNF expression leads to antidepressant effects against subsequent stressful situations. There are three possibilities as to why HSP105 increases BDNF: (i) HSP105 may regulate enzymes that modulate the conversion of pro-BDNF to BDNF; (ii) HSP105 may stabilize BDNF protein formation; and (iii) HSP105 may activate BDNF transcription factors, such as adenosine 3′,5′-monophosphate response element–binding protein (CREB). In general, if the level of transcription is increased, transcription factors are activated. According to hypothesis (i) or (ii), BDNF protein expression will increase regardless of *BDNF* mRNA levels. However, the present study showed that *BDNF* mRNA levels were significantly increased by GGA administration in the mouse hippocampus and HT22 cells. These results suggest that HSP105 can directly or indirectly regulate BDNF transcription factors. If HSP105 acts directly, HSP105 may control transcription of BDNF via CREB, a key transcription factor for BDNF induction (33). In contrast, if HSP105 acts indirectly, HSP105 may regulate factors that activate the transcription of BDNF, such as mitogen-activated protein kinase (MAPK). Activation of the MAPK pathway can also regulate transcription through phosphorylation of CREB. Here, we observed that GGA treatment increased the mRNA levels of *BDNF* but not *NGF*, *NT-3*, and *CNTF*. The genes that regulate CREB are well known. However, detailed knowledge on which transcription factors induce CNTF, NT-3, and NGF production is lacking. Moreover, we also investigated phospho-CREB expression in mouse hippocampus but found no significant changes between stressed mice and GGA-stressed mice (fig. S5). It has been reported that not only CREB but also CaRF (calcium-responsive transcription factor), USF1/2 (upstream transcription factor 1/2), BHLHB2 (basic helix-loop-helix B2 transcription factor), NFκB (nuclear factor κB), and NFAT (nuclear factor of activated T cells) are related to *BDNF* transcription (34). Therefore, our findings suggest that other transcription factors are involved in the GGA-induced increase of *BDNF*. Thus, identification of the transcription factors that regulate the expression of neurotrophins is needed.

Neurotrophins bind to two classes of receptors, p75 and the tropomyosin receptor kinase (trk) receptors. The trkA receptor displays high affinity for NGF, which induces the activation of the phosphatidylinositol 3-kinase (PI3K)–Akt pathway and MAPK pathway. BDNF

and NT-3 bind to trkB receptors and activate the MAPK pathway, PI3K-Akt pathway, and phospholipase C–γ–Ca$^{2+}$ pathway. K252a inhibits the trk family of receptor tyrosine kinases and other serine/threonine protein kinases (35). Here, we demonstrated that K252a inhibited the GGA-mediated antidepressant effect. Furthermore, GGA elicited an increase in HSP105, and an increase in *BDNF*, but not *NGF*. These results support the hypothesis that GGA-mediated HSP105 may modulate BDNF expression, which binds to trk receptors to rescue depression-like behavior. To clarify whether HSP105 modulates BDNF expression, we performed HSP105 knockdown. Here, we demonstrated for the first time that HSP105 promotes BDNF expression in the hippocampus, which results in the amelioration of depression-like behavior. The effect of HSP105-siRNA was examined in HT22 cells and the mouse brain. In HT22 cells, HSP105 knockdown significantly decreased *BDNF* mRNA expression. In addition, HSP105-siRNA aggravated the GGA-mediated antidepressant effects in vivo. Only the sucrose preference test showed no significant changes after HSP105-siRNA treatment. Thus, we hypothesize that HSP105 may only have a slight effect in anhedonia-like behavior or, alternatively, that the effect of HSP105 knockdown may be insufficient to cause a change in anhedonia behavior. In contrast, increased immobility time in the tail suspension test was significantly inhibited by HSP105 knockdown. These results suggest that the effects of HSP105 on hippocampal proliferation and antidepressant effects are most likely mediated through increases in BDNF expression. The decrease in hippocampal BDNF levels is correlated with depression (21), and infusion of BDNF into the hippocampus induces antidepressant effects in rodents (24).

GGA has always been considered a gastrointestinal drug for protecting the mucosa. Here, we demonstrate that GGA reduced depression-like behavior and BDNF expression in the stressed mouse hippocampus. GGA can cross the blood-brain barrier (BBB), as indicated here by the increase in *HSP105* mRNA expression in the hippocampus following oral administration. This is further supported by Mikuriya et al. (36), because GGA is a lipid-soluble agent that easily crosses the BBB. In terms of drug dosage, 0.34% (w/v) GGA is a rather high concentration compared with a clinical dose in humans (150 mg, three times a day; approximately 1.5 to 3 mg/kg) (37). The median lethal dose (LD$_{50}$) of GGA is more than 15,000 mg/kg by oral administration in rodents (38). The dose chosen in this study was determined using other reports that used GGA (800 mg/kg) for mice (31). Here, GGA showed no adverse effects at a dose of 0.34% (w/v). Other reports also showed that GGA had no adverse effects at high doses [800 mg/kg, or 0.25, 0.5, 1, and 2% (w/v)] in rodents (5, 31). In the FAERS database, results suggested that the clinical dose in humans could help prevent the onset of drug-induced depression. Here, we focused on medications known to cause drug-induced depression. Peginterferon α-2a or peginterferon α-2b is usually used for the treatment of chronic hepatitis C. Ribavirin is also used as an antiviral drug for hepatitis C infection and is often given in combination with peginterferon α-2a or peginterferon α-2b. We observed that the reporting of depressive events decreased when teprenone (GGA) was used in combination with peginterferon or ribavirin. The ROR of peginterferon or ribavirin was 1.82. These results suggest that if the patient used either peginterferon α-2a, peginterferon α-2b, or ribavirin, the depressive side effects were likely to occur 1.82 times more when compared with other medications. Meanwhile, coadministration of teprenone (GGA) with peginterferon or ribavirin reduced ROR to 1.13, indicating that teprenone (GGA) suppressed the depressive side effects. We also observed gender differences in depression—female cases with depression were much higher than male cases (Table 1).

We cannot rule out limitations of our FAERS analysis and, in particular, the reported drug side effects. Further experiments are needed for depressive patients with or without teprenone.

In summary, we demonstrate for the first time the possibility of using GGA to ameliorate depression via the HSP105-BDNF pathway. These observations suggest that GGA-mediated HSP induction may provide a novel therapeutic strategy for depression-like behavior.

## MATERIALS AND METHODS

All animal procedures were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the Japanese Association for Laboratory Animal Science. All experiments were approved by the Animal Care and Use Committee of the Okayama University of Science. According to these guidelines, all efforts were made to minimize the number of animals used and their discomfort.

### Animals
Six-week-old male C57BL/6 mice (not siblings, purchased from Shimizu Experimental Animals) were housed in the Animal Research Center of Okayama University of Science at a controlled ambient temperature of 22°C with 50 ± 10% relative humidity and a 12-hour light/dark cycle (lights on at 7:00 a.m.). They were fed a normal chow diet and water ad libitum for a 2-week habituation. All procedures were approved by the institutional ethics committee (authorization numbers 20130205-1 and 2016-03).

### Social defeat stress exposure
Social defeat stress was carried out using a previously reported method (20). Test mice were exposed to a different CD1 strain aggressor mouse each day for 10 min for a total of 15 days. After 10 min of physical contact, test mice were separated from the aggressor and placed across a plastic separator with holes, where they remained in sensory contact with the CD1 aggressor for the remainder of the 24 hours. Each day, experimental mice were exposed to a new resident home cage. Controls were housed individually. After the last defeat, a social interaction test was performed to measure the behavioral consequences of the chronic defeat stress.

### GGA treatment
GGA and gum arabic were purchased from Wako Inc. For oral administration to mice, GGA granules were emulsified in 5% (v/v) gum arabic at a concentration of 0.34% (w/v). The dosage of GGA was selected on the basis of the effective concentration response (800 mg/kg per day) (31). Briefly, 8- to 10-week-old mice had an average body weight of 25.5 g and an average daily water consumption of 6 ml, which was why a concentration of 0.34% (w/v) was chosen. GGA was administrated to mice from 8 weeks old until the end of the stress exposure. The control and stressed mice group received drinking water supplemented with 5% (w/v) gum, which was used as a vehicle for GGA.

K252a (25 μg/kg, Sigma), a broad-spectrum tyrosine kinase antagonist, was used (35). To avoid any unwanted effects from K252a, we intraperitoneally administered a low concentration of K252a (39) 15 days during the stress exposure.

### Immunostaining
Brains were collected and immunostained, as previously described (40). Briefly, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and transcardially perfused with 0.9% (w/v)

saline, followed by 4% (w/v) paraformaldehyde and 0.35% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (PB) (pH 7.4). The brains were postfixed overnight in 4% (w/v) paraformaldehyde in 0.1 M PB and cryoprotected in 15% (w/v) sucrose in 0.1 M PB with 0.1% (w/v) sodium azide overnight at 4°C. The brains were sectioned (20 μm) using a cryostat, and immunohistochemistry was performed on free-floating sections. Every 12th section was incubated with 2 M HCl in 0.1 M PB for 30 min at 65°C to block endogenous peroxidase. Sections were then rinsed with 10 mM phosphate-buffered saline (PBS; pH 7.4) and incubated for 1 hour in PBS containing 10% (v/v) normal goat serum containing 1% (w/v) bovine serum albumin (BSA) at room temperature. Thereafter, sections were incubated in a rabbit anti-DCX antibody (1:1000; Abcam plc), rabbit anti-NeuN (1:1000, Merck Millipore Co.), or rabbit anti-BDNF (1:300, Abcam) overnight at 4°C. After washing, sections were incubated for 60 min at room temperature with Alexa Fluor 488 goat anti-rabbit immunoglobulin G (Life Technologies) and diluted 1:400 in PBS containing 1% (w/v) BSA. Following several washes in PBS, sections were coverslipped with glycerol/PBS (1:1, v/v) and observed under a fluorescent microscope (EVOS FLoid Cell Imaging Station, Life Technologies). All morphological analyses were performed on blind-coded slides. The total number of DCX- or NeuN-labeled cells in the dentate gyrus was counted for each section. BDNF-positive cells were evaluated by density of area per region using ImageJ (https://imagej.nih.gov/ij/). To evaluate neurogenesis, we examined DCX-positive cells in every 12th bilateral section (240-μm interval). Cells throughout the granular zone were counted. The number of DCX- or NeuN-positive cells was multiplied by a factor of 12 to estimate the total number of each positive cell type.

## Behavioral assessments
### Open-field test
Spontaneous locomotor activity was evaluated using an open-field test, as described previously (40). Mice were individually placed in the center of a circle open-field chamber (diameter, 57.5 cm; height, 32 cm). All movements were recorded using a digital camera for 3 min. The floor was divided into 19 sections, with each section of approximately the same area. Briefly, two circles (13.5 and 35.5 cm in diameter) were drawn, focusing on the center of the chamber. A line was drawn radially and divided into 18 equal parts. Each section was 13.5 cm in diameter. The 35.5-cm-diameter circle was defined as the center. Locomotor activity was scored when a mouse removed all four paws from one section and entered another. The line crossings, rearing (frequency with which the mice stood on their hind legs), and time spent in the center were measured over the course of 3 min using a counter or stopwatch. The apparatus was cleaned after each trial.

### Social interaction test
Mice were placed in a new area (a 46 cm × 36 cm white plastic open field) with a small-animal cage at one end and observed for 2.5 min in the absence of another mouse, followed by 2.5 min in the presence of a caged, unfamiliar target CD1 mouse. Social interaction was quantified by stopwatch by comparing the amount of time that the test mouse spent in the interaction zone near the small-animal cage in the presence versus the absence of the target CD1 mouse.

### Forced swim test
Animals were placed in a 1-liter Griffin beaker [15 cm (height) × 11 cm (diameter); filled with 28°C water to a depth of 10 cm] for 6 min, and the duration of floating (that is, the time during which the animal made only small movements necessary to keep its head above water) and the latency to the immobility time were scored. Immobility time was analyzed during the last 5-min period of the test. The animals were then dried and returned to their home cage.

### Tail suspension test
The tail suspension test was performed on the basis of a previous method (41). The mouse was hung 15 cm above the floor by the tip of the tail (1 cm) and was adhered to an aluminum bar. The total test procedure of mouse immobility time was counted during a test period of 6 min (1 min of adaptation time and 5 min of recording). A mouse was considered immobile when it was passively suspended and completely motionless.

### Sucrose preference test
The test was based on methods described by Uchida et al. (42). Animals were habituated to drinking water from two bottles for 2 days following the last defeat exposure. In the sucrose preference test, two preweighed bottles [one containing tap water and the other containing a 1% (w/v) sucrose solution] were presented to each animal for 4 hours. The position of the water and sucrose bottles (left or right) was switched every 2 hours. The bottles were weighed again, and the weight difference represented the animal's intake from each bottle. The sum of water plus sucrose intake was defined as the total intake, and sucrose preference was expressed as the percentage of sucrose intake relative to the total intake.

## RNA extraction
The animals were sacrificed on day 16 by administration of an overdose of sodium pentobarbital (100 mg/kg). Total RNA was extracted from the hippocampus, placed in RNAlater (Life Technologies), and stored at −30°C. Total RNA was extracted using the RNeasy Plus Micro Kit (Qiagen). At the end of the extraction, RNA samples were dissolved in nuclease-free water (Qiagen), and the optical density values of each sample were determined using an absorption meter (Shimadzu Co.). We performed reverse transcription using Moloney murine leukemia virus (Wako Pure Chemical Industries Ltd.) according to the manufacturer's protocol. Specificity of amplification was verified by the monophasic characteristic of the melting curve generated for each amplification product by the Eco Real-Time PCR System (Illumina Inc.) at the end of a PCR.

## Quantitative analysis by real-time PCR
The reverse-transcribed mixture was used as a template for subsequent real-time PCR. Real-time PCR was performed according to the KAPA SYBR Fast qPCR kit (Nippon Genetics Co. Ltd.) and analyzed with an Eco Real-Time PCR System (Illumina Inc.). Primers, designed by the authors, were based on the coding sequences of mouse genes deposited in GenBank. The data were analyzed with the mean threshold cycle equation. Primer information is shown in table S1. GAPDH served as an internal control. The threshold cycle values for both the target (HSPs or neurotrophins) and internal control (GAPDH) genes were determined. The fold change of each gene was normalized to GAPDH and, relative to the expression in control samples, calculated for each sample.

## Western blotting
For Western blot analysis, collected hippocampi were placed in RNAlater (Life Technologies) and homogenized in an SDS sample buffer. Protein extracts were separated by SDS–polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Hybond P; GE Healthcare). The membrane was blocked with a blocking agent (GE Healthcare) and then incubated at 4°C overnight with a primary antibody for rabbit anti-HSP110 (1:5000, StressMarq Biosciences Inc.),

rabbit anti-HSP25 (1:5000, Cosmo Bio Co. Ltd.), mouse anti-HSP60 (1:1000, Cosmo), mouse anti-HSP72 (1:1000, Cosmo), mouse anti–phospho-CREB (Ser[133]) (1:5000, Cell Signaling Technology), rabbit anti-CREB (Ser[133]) (1:5000, Cell Signaling), and rabbit-BDNF (1:5000, Abcam). After washing with tris-buffered saline containing 0.1% (v/v) Tween 20, the membranes were incubated with horseradish peroxidase–conjugated secondary antibody (1:20,000) for 1 hour at room temperature. The antibody-reactive bands were visualized using the chemiluminescent substrate kit (GE Healthcare). Bands were analyzed by densitometry using FluorChem 8800 (Alpha Innotech), and the content of GAPDH, which was detected using a rabbit anti-GAPDH antibody (1:20,000; Sigma), was used as a control to ensure that the same amount of protein was loaded in each lane.

### siRNA constructs
The siRNA reagents used were Dharmacon Mouse ON-TARGET-plus SMARTpool siRNA and Mouse ON-TARGETplus Non-targeting siRNA (GE Healthcare Dharmacon Inc.).

### Neuronal cell culture and transfection
The mouse hippocampal neuronal cell line HT22 was a gift from H. Kimura (National Institute of Neuroscience, Japan). HT22 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere containing 5% (v/v) $CO_2$ and 95% air. The cells were seeded in 12-well plates at a density of $0.2 \times 10^5$ cells per well. Twenty-four hours after being seeded, the medium was replaced with antibiotic-free medium. HT22 cells were transfected using Lipofectamine RNAiMAX reagent (Life Technologies). The siRNA-lipid complex (10 pmol) was added to cells and incubated for 24 hours. The medium was changed 24 hours after transfection to DMEM containing penicillin/streptomycin and 10% (v/v) FBS. Cells were incubated with 50 μM GGA or vehicle [0.5% (v/v) ethanol–containing medium] for 22 hours following heat shock at 41°C for 2 hours with 50 μM GGA or vehicle. GGA was dissolved in 100% (v/v) ethanol. Following incubation, the cells were washed with PBS buffer and lysed with RNAlater, and mRNA expression was evaluated by real-time PCR.

### Intracerebroventricular injection
Mice were divided into three groups: (i) vehicle [5% (v/v) gum arabic], (ii) HSP105-siRNA intracerebroventricularly (ICV) with 0.34% (w/v) GGA, (iii) and nontargeting control siRNA ICV with GGA on days 1, 7, and 14 during a 15-day stress exposure. Inhaled diethyl ether was used for brief anesthesia during ICV injections. The siRNA transfections used in vivo-jetPEI reagent (Polyplus-transfection Inc.). siRNA-polyethylenimine complex administration was performed by direct injection into the right lateral ventricle through the intact scalp aiming at 1 mm posterior to bregma and 1 mm right from the midline, as described previously (40, 43).

### FAERS data sources
Adverse events recorded from the third quarter of 2010 to the second quarter of 2015 in the FAERS database were downloaded from the FDA website (www.fda.gov). A total of 2,326,853 reports, which excluded reports with unknown age or gender, were obtained. The drug selected for this investigation was teprenone (GGA pharmaceutical drug name). We chose data where GGA was used as a concomitant drug in patients aged 20 to 79 years. We investigated the effect of com-bination treatment with GGA and interferons (peginterferon α-2a, peginterferon α-2b, and ribavirin), which are well known as drugs that induce depression (25, 26). All reported adverse events of interest (depression, major depression, agitated depression, or depression suicidal) were defined as "cases," and all reported other adverse events were defined as "non-cases." The ROR was used as the signal score, which was calculated using a case/non-case method (44, 45). A 2 × 2 contingency table was the framework for analysis (table S2). We calculated signal scores to evaluate whether GGA was associated with an adverse event.

### Statistical analysis
All data are expressed as means ± SEM. GraphPad Prism 5 software (GraphPad Software Inc.) was used for all statistical analyses. Comparisons between two values were analyzed using one-tailed Student's $t$ test. ANOVA followed by Tukey's multiple comparison test was used to determine statistical significance, where appropriate. A $P$ value of <0.05 was considered statistically significant. Two-way ANOVAs were also performed: If a statistical interaction was observed between factors, comparison of all four groups was performed by Tukey's post hoc test. Statistical differences are indicated by different letters ($P < 0.05$). If no significant interaction was observed, statistical effects of one or both factors are denoted as an inset for each group.

### SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/5/e1603014/DC1

fig. S1. Effect of stress or GGA on mature neurons in the mouse hippocampus.
fig. S2. Effect of stress or GGA on BDNF distribution in the mouse hippocampus.
fig. S3. Effect of GGA or GGA + K252a on mouse behavior with nonstress conditioning.
fig. S4. Effect of HSP105-siRNA on expression of HSP25, HSP60, and HSP72 in the nonstressed mouse hippocampus ($n = 4$ animals per group).
fig. S5. Effect of GGA on phospho-CREB expression in the stressed mouse hippocampus (stress + VEH, $n = 5$; stress + GGA, $n = 6$ animals).
table S1. Oligonucleotide sequences for real-time PCR amplification.
table S2. A 2 × 2 table used for the calculation of ROR.

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