Wild Ginseng Attenuates Anxiety- and Depression-Like Behaviors During Morphine Withdrawal

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The purpose of this study was to evaluate whether wild ginseng (WG) administration could attenuate anxiety- and depression-like behaviors and expression of corticotrophin-releasing factor (CRF) and neuropeptide Y (NPY) following withdrawal from repeated morphine administration in rats. Male rats were administered daily doses of WG (50, 100, or 200 mg/kg, i.p.) for 5 days, 30 min before morphine injection (40 mg/kg, s.c.). The anxiety- and depression-like behavioral responses were measured 72 h after the last morphine injection using an elevated plus maze (EPM) and forced swimming test (FST), respectively. Changes in hypothalamic CRF and NPY expressions were also examined by analyzing their immunoreactivities in the hypothalamus. Daily administration of WG significantly reduced anxiety- and depression-like behavior, and elicited the suppression of CRF expression and the stimulation of NPY expression in the hypothalamus. Our results demonstrated that WG extract might be effective at inhibiting the anxiety and depression responses due to morphine withdrawal by possibly modulating the hypothalamus CRF and NPY systems. Furthermore, these findings imply that WG extract can be used for developing new medication to cure or alleviate morphine withdrawal symptoms and to prevent relapses of morphine use.

Keywords: Morphine, wild ginseng, anxiety, depression, neuropeptide Y, corticotrophin-releasing factor

Morphine, a strong pain reliever, is widely used to treat moderate to severe pain and a number of other pathological indications. This said, the abuse of morphine and its subsequent withdrawal cause psychiatric side-effects, including anxiety and depression [20]. Many studies have demonstrated that morphine withdrawal causes anxiety- and depression-related disorders in humans and corresponding behavioral responses in animals [1, 21]. Anxiety and depression associated with morphine withdrawal can be alleviated by the administration of antidepressant or anxiolytic drugs, such as fluoxetine or agmatine [28]. However, some antidepressants exert undesirable side-effects, such as drowsiness, dryness of the mouth, headache, nausea, and sexual dysfunction [8].

Recent studies suggested that Panax ginseng (PG) was found to reduce depression symptoms and anxiety disorders in humans [2]. Some studies have reported that PG showed antidepressant-like activity in the forced swimming test (FST) and also reduced anxiety-like behavior in the elevated plus maze (EPM) test in an animal model [4, 27]. Wild ginseng (WG) is the ginseng (the root of Panax ginseng C.A. Mayer) that naturally grows in the mountains and is distinguished from field-cultivated ginseng. It is known to have more pharmacological efficacy and is thus more expensive than cultivated ginseng. In this study, WG indicates the ginseng that has grown undisturbed in the Korean forest for many years from the seeds initially scattered by humans [9]. In terms of seeding methods, it is also differentiated from truly wild ginseng of which the seeds have been distributed through natural vectors such as birds.

Until now, there are still unresolved questions about the mechanisms underlying WG’s effect as a therapeutic intervention for treating psychiatric side-effects, including the withdrawal symptoms associated with morphine use. The effects of WG on morphine withdrawal-induced anxiety- and depression-like behavioral alterations have not been examined in animal models. In the present study, the pharmacological effects of WG extract on anxiety- and depression-related behaviors following repeated morphine administration and withdrawal were investigated. Morphine withdrawal-induced behaviors were examined using the EPM and the FST. We also tried to elucidate the underlying mechanism of the effect of WG administration on morphine use.

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dependency regarding the alterations of CRF and NPY expressions in the hypothalamus of the rat brain.

**Materials and Methods**

**Animals**

Adult male Sprague-Dawley (SD) rats weighing 260–280 g were obtained from Samtaco Animal Co. (Seoul, Korea). The rats were housed in a limited-access rodent facility with up to five rats per polycarbonate cage. The room controls were set to maintain the temperature at 22°C ± 2°C and the relative humidity at 55% ± 15%. Cages were lit by artificial light for 12 h each day. Sterilized drinking water and standard chow diet were supplied ad libitum to each cage during the experiments. The animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised in 1996, and were approved by the Kyung Hee University Institutional Animal Care and Use Committee. All animal experiments began at least 7 days after the animals arrived.

**Preparation of the Drugs and the Methanol Extracts of WG and PG**

Wild ginseng roots (adventitious root culture of *Panax ginseng*, WG) were collected in Chonbuk Province in Korea and purchased from Baekjesansam Co. (Mr. Jong-Gu Lee, Jinan-kun, Jinan-up, Yeonjang-Ri #45-1, Chonbuk, 567-807, Korea). PG (*Panax ginseng*) was purchased from Dongwoodang Pharmacy Co., Ltd (Yeongheon, Korea).

Voucher specimens of WG and PG have been deposited at the herbarium located at the College of Oriental Medicine, Kyung Hee University (No. KH-WG01 for WG and No. KH-PG01 for PG). WG and PG (100 g each) were cut into small pieces and extracted three times with 21 of 80% methanol by sonication in a reflux condenser for 24 h at room temperature (25 ± 2°C), respectively. The solutions were combined, filtered through Whatman No. 1 filter paper, concentrated using a rotary vacuum evaporator (Rotavapor R-124; BÜCHI Labortechnik AG, Flawil, Switzerland) under reduced pressure, refrigerated in a recirculating chiller (EYELA CCA-1124; Tokyo Rikakikai Co., Tokyo, Japan) to obtain concentrated extracts, and then lyophilized (EYELA FD-800; Tokyo Rikakikai Co., Tokyo, Japan). The yields of aqueous phases of WG and PG were 11.6% and 20.6% (w/w), respectively. Morphine hydrochloride (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was obtained from the standard commercial suppliers. Morphine hydrochloride was dissolved in 0.9% saline solution.

**Morphine Treatment and Experimental Groups**

The withdrawal group following repeated morphine administration was given morphine (40 mg/kg-body weight, s.c., MOR group, n=6) twice a day for 5 consecutive days. No drugs were injected within 72 h after the last morphine injection, and behavioral responses were tested during this period. The vehicle-treated rats (as a negative control of the addiction withdrawal model development) were administered with saline (0.9% NaCl, s.c.) instead of morphine in the same way (SAL group, n=6). The WG- or PG-treated groups were divided as follows: 50 mg/kg WG plus morphine-treated group (WG50+MOR, n=6), 100 mg/kg WG plus morphine-treated group (WG100+MOR, n=6), 200 mg/kg WG plus morphine-treated group (WG200+MOR, n=6), and 500 mg/kg PG plus morphine-treated group (PG500+MOR, n=6). The WG or PG treatments were given intraperitoneally 30 min prior to the injection of morphine for 5 consecutive days, as the development phase. The anxiety- and depression-like behavioral responses were measured 72 h after the last morphine injection in all groups using the EPM and FST tests. The experimental schedule of all drug administration and behavioral tests are shown in Fig. 1.

**Measurement of Elevated Plus Maze (EPM)**

The EPM test has been used to assess internal conflict between voluntary approaches and withdrawal tendencies as a rodent model for human anxiety [18]. Because this test is based on a natural fear of open and elevated spaces, the number of entries into the open arms and the time spent in open arms are negatively correlated with the anxiety level of the subject. The EPM consists of a plus-shaped plastic apparatus with two open and two enclosed arms, each with an open roof. The apparatus was painted with black enamel and was elevated 50 cm from the floor. All arms were 10 cm in width and 50 cm in length and joined at the center to form a 10 cm² central platform. Two closed arms opposite each other were surrounded by 40 cm high plastic walls with an open roof, and the other two open arms were without any walls. Exploration of the open arms was encouraged by testing under indirect dim light (2×60 W).

At the start of testing, the rat was placed on the central platform facing the given open arm, and was allowed to move freely for 5 min. Each rat was placed in the center of the maze, after which the cumulative time spent on each arm and the numbers of entries into the open or closed arms were recorded during a 5 min test session 72 h after the last injection of morphine or saline. Entry into an arm was defined as beginning when the animal placed all four paws in that arm. The maze was cleaned with alcohol after each rat was tested. The behavior in the maze was recorded using a video camera mounted on the ceiling above the center of the maze and was relayed to the S-MART program (PanLab, Barcelona, Spain), which included a standard measure of anxiety-like behaviors, and several additional assessments of open-arm exploration. The standard measure of anxiety was indicated by the time ratio, which is the time spent in the open arms of the maze divided by the total time spent in any arm of the maze. A smaller ratio indicates less open-arm exploration or more “anxiety.” Additional measures of activity and exploration included head dips and rearing, which were independently scored in the closed arms, open arms, and central
Measurement of Forced Swimming Test (FST)
In the FST, the effect of WG on depression-like behavior produced by withdrawal from repeated morphine injection was evaluated using the method described by Porsolt et al. [19] and with some modifications made by others [11]. Briefly, each rat was individually placed in a plastic cylinder (20 cm diameter × 40 cm height) containing 30 cm of water at about 25°C, so rats could not support themselves by touching the bottom with their feet. Two swimming sessions were conducted: an initial 15 min pretest followed 24 h later by a 6 min test. The duration of immobility was scored during the last 4 min of the 6 min test period. The animals’ behavior was continuously recorded throughout the testing session with an overhead video camera. Following both swimming sessions, the rats were removed from the water, and then placed into their original cages after being warmed and dried. Activity was defined as the swimming, jumping, diving, or scratching of the walls. Immobility was defined when floating motionless or by making only those movements necessary to keep the rat’s head above the water.

Corticotrophin-Releasing Factor (CRF) and Neuropeptide Y (NPY) Immunohistochemistry
For immunohistochemical studies, the animals were deeply anesthetized with sodium pentobarbital (80 mg/kg, by intraperitoneal injection) and perfused through the ascending aorta with normal saline (0.9%) followed by 300 ml (per rat) of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brains were removed, post-fixed overnight, and cryoprotected with 20% sucrose in 0.1 M PBS at 4°C. Coronal sections (30 mm thick) were cut through the hypothalamus using a cryostat (Leica CM1850; Leica Microsystems Ltd., Nussloch, Germany). The sections were obtained according to the rat atlas of Paxinos and Watson [17]. The sections were immunostained for CRF and NPY expressions using the avidin-biotin-peroxidase complex (ABC) method. Briefly, the sections were rinsed three times for 5 min each in PBS and then incubated with primary goat anti-CRF antibody (1:2,000 dilution; Santa Cruz Biotechnology Inc., California, USA) and rabbit anti-NPY antibody (1:2,000 dilution; Immunostar Inc., Hudson, WI, USA) in PBST (PBS plus 0.3% Triton X-100) for 72 h at 4°C. The sections were washed for 5 min in PBS and then incubated for 120 min at room temperature with biotinylated rabbit anti-goat IgG antibody serum for anti-NPY antibody. Both secondary antibodies were obtained from Vector Laboratories Co. (Burlingame, CA, USA) and diluted 1:200 in PBST containing 2% normal serum. To visualize immunoreactivity, the sections were incubated for 90 min in ABC reagent (Vectastain Elite ABC kit; Vector Labs. Co., Burlingame, CA, USA), washed three times for 5 min in PBS, and incubated in a solution containing 3,3′-diaminobenzidine (DAB; Sigma) and 0.01% H2O2 for 1 min. Finally, the tissues were washed in PBS, followed by a brief rinse in distilled water, and mounted individually onto slides. Slides were allowed to air dry and were then cover-slipped. Images were captured using the AxiosVision 3.0 imaging system (Carl Zeiss, Inc., Oberkochen, Germany) and processed using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA). The sections were viewed at 100× magnification, and the numbers of cells within 100 × 100 μm2 grids were counted by observers blinded to the experimental groups. Hypothalamus area cells were obtained according to the stereotactic atlas of Paxinos and Watson [17]. The cells were counted in three sections per rat within the hypothalamus area.

Statistical Analysis
The experimental results were expressed as the mean ± standard error (SE). The behavioral data were calculated and analyzed by repeated measures analysis of variance (ANOVA) using SPSS (Version 13.0; SPSS Inc., Chicago, IL, USA). The statistical significance of the differences among groups was further analyzed using Tukey’s post-hoc test. Immunohistochemical data were also analyzed by one-way ANOVA followed by Tukey’s post-hoc test. In all analyses, p < 0.05 was considered significant.

RESULTS
Effect of WG on Morphine-Induced Anxiety-Like Behavior
Anxiety expressed by a decrease in open-arm exploration in the EPM test was analyzed. Rats were challenged in the EPM test at 72 h after the last injection of morphine or saline (Fig. 2). Student’s t tests showed that morphine withdrawal significantly reduced both the percentage of time spent (t(10)=2.599; p<0.05) and the percentage of entries (t(10)=2.973; p<0.05) in the open arms of the maze, indicating an anxiolytic-like effect (Fig. 2A and 2B, respectively). On the other hand, statistical analysis revealed that withdrawal did not significantly change the number of entries in the closed arms (t(10)=1.368; p=0.214; data not shown).

The effects of administration of WG prior to MOR injection were evaluated during the withdrawal period using the EPM test. Statistical analyses of behavioral data from the EPM test showed that the percentage of time spent [F(4,29)=8.106, p<0.001] and the percentage of entries [F(4,29)=8.189, p<0.001] into the open arms of the maze revealed significant differences among the six experimental groups (Fig. 2C and 2D, respectively). Post-hoc comparisons identified a significantly decreased percentage of time spent in the open arms of the maze at 72 h after the last morphine injection, as compared with the SAL group (p<0.05). However, the rats in the WG200+MOR groups showed a significantly greater percentage of time spent in the open arms of the maze, as compared with those in the MOR group (p<0.05), whereas the WG100+MOR group did not show any the significant difference (Fig. 2C).

Similarly, post-hoc comparisons identified a significantly decreased percentage of entries in the open arms of the maze at 72 h after the last morphine injection, as compared with the SAL group (p<0.05). However, the rats in the WG200+MOR groups showed a significantly greater percentage of entries in the open arms of the maze, as compared with those in the MOR group (p<0.05) (Fig. 2D). Because no significant group differences of the number
were observed in closed-arm entries in the EPM test [F(4,29)=0.106, p=0.979], it could be suggested that the observed anxiety-like behaviors of morphine-treated rats were not attributed to differences in locomotion activity (Fig. 2D). The percentage of time spent and entries in the open arms of the maze of the WG200+MOR group were both higher than those in the PG500+MOR group.

**Effect of WG on Morphine-Induced Depression-Like Behavior**

Following withdrawal from repeated MOR exposure, rats exhibited a markedly “depressed” phenotype, characterized by increased immobility time during the FST, as compared with saline-treated controls. Rats were subjected to the FST at 72 h after the last injection of morphine or saline (Fig. 3). Immediately after the last morphine administration (day 1), a decrease in immobility was recorded, as compared with the SAL group. However, on day 2 following withdrawal from morphine administration, an opposite phenomenon, namely the enhancement of immobile time without any influence on ambulatory activity, was observed (p<0.05 versus SAL group, Fig. 3A). The phenomenon persisted for at least another 3 days (p<0.01).

The effects of administration of WG prior to MOR injection were evaluated during the withdrawal period using the FST. Statistical analysis of behavioral data from

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**Fig. 2.** Changes of anxiety behaviors in the elevated plus maze on different days after withdrawal from repeated saline or morphine administration. Rats were placed in the center of the maze facing an enclosed arm and their behaviors recorded for 5 min at 24, 48, and 72 h following the last saline or morphine injection. The percentage of time spent in open-arm exploration (A) and the percentage of entries into open arms (B) in the elevated plus maze are shown. Effect of WG on the percentage of time spent in open-arm exploration (C) and the entries into open arms (D) during morphine withdrawal are presented. The withdrawal group following repeated morphine administration was given morphine (40 mg/kg-body weight, s.c., MOR group, n=6) twice a day for 5 consecutive days. The vehicle-treated rats were administered with saline (0.9% NaCl, s.c.) instead of morphine in the same way (SAL group, n=6). The WG- or PG-treated groups were divided as follows: 50 mg/kg WG plus morphine-treated group (WG50+MOR, n=6), 100 mg/kg WG plus morphine-treated group (WG100+MOR, n=6), 200 mg/kg WG plus morphine-treated group (WG200+MOR, n=6), and 500 mg/kg PG plus morphine-treated group (PG500+MOR, n=6). Data were analyzed using repeated measures ANOVA following by Tukey’s test. *p < 0.05 vs. SAL group; # p <0.05 vs. MOR group. Vertical bars indicate SE.
the FST showed that the time spent immobile in the maze significantly differed among the six experimental groups \(F(5,41)=3.046, p<0.05\). Post-doc comparisons identified that immobility time in the maze significantly increased at 72 h after the last morphine injection, as compared with the SAL group \(p<0.05\), Fig. 3B). However, the rats in the WG200+MOR groups showed a significantly decreased immobile time during the 5 min of the FTS, as compared with the MOR group \(p<0.05\), indicating that administration of WG, but not the administration of PG, decreased depression-like behavior. The time spent immobile in the FST of the WG200+MOR group was lower than that in the PG500+MOR group.

**Effect of WG on Morphine-Induced CRF-Like Immunoreactivity**
Following withdrawal from the repeated injections of morphine, CRF-like immunoreactivity was detected primarily...
in the cell bodies of hypothalamic regions, including the PVN (Fig. 5). In brains of the MOR group, the numbers of CRF immunoreactive fibers in the PVN was increased by 209.05%. Comparison of the numbers of CRF-immunoreactive neurons using one-way ANOVA revealed a significant difference among the groups \[F(5,125)=16.148, p<0.001\]. The post-hoc comparisons revealed that rats treated with MOR alone showed a greater increase in CRF expression, as compared with the SAL group (p<0.001). The numbers of CRF-like immunoreactive cells in PVN were 10.52±1.61 (100±5.20%) in the SAL group, 22.00±1.98 (209.05±18.81%) in the MOR group, 17.05±0.98 (161.99±9.35%) in the WG50+MOR group, 17.14±1.49 (162.90±14.19%) in the WG100+MOR group, 14.52±1.89 (138.01±17.94%) in the WG200+MOR group, and 16.86±0.99 (160.18±9.39%) in the PG500+MOR group. The CRF expression of the WG200+MOR group was lower than that in the PG500+MOR group.

**DISCUSSION**

The present results demonstrated that withdrawal from repeated administration of morphine increased anxiety- and depression-like behaviors in rats. In addition, daily morphine injections increased CRF and decreased NPY expressions in the PVN of rat hypothalamus. Our results showed that administration of WG significantly reduced anxiety- and depression-like behaviors during morphine withdrawal by modulating the CRF and NPY systems in the hypothalamus. Thus, the results of our study support the possibility that WG might have anxiolytic and antidepressant effects. In this study, on the third day (day 3) after repeated morphine administration for 5 consecutive days, a significant decrease in exploring time in the open arms of the EPM and an increase in the immobility in the FST were observed as compared with saline-treated controls. However, only small differences in EPM and FST behavioral tests were
observed on days 1 and 2 after repeated morphine administration. The third day (day 3) after repeated morphine administration was thus found to be the optimal time to observe changes of withdrawal-related behavior.

Morphine abuse continues to be a serious risk factor for various problems in our society, and a need exists to develop effective medication that can treat morphine withdrawal symptoms, particularly with regard to relapse into drug-seeking behavior. Previous studies have confirmed that withdrawal from morphine administration elicits both anxiety- and depression-like behaviors in rats, as assessed in the elevated plus maze [5], and the forced swim test [26]. In the present study, withdrawal from morphine administration also significantly increased the anxiety- and depression-like behaviors attributed to morphine discontinuation in rat models in a similar manner to that elucidated in previous studies [5, 26].

Many studies have validated the EPM test as an appropriate measure of anxiety in animal models [15, 22, 25]. When an animal was confronted with a new threatening situation, the time spent in open arms during the EPM test decreased, which indicates an increase of anxiety. In the present study, WG administration prior to repeated injections of morphine significantly reduced the number of entries and the time spent in open arms by morphine withdrawal. This inhibition reflects an anxiolytic activity of WG extract. Anxiolytic agents, including selective serotonin reuptake inhibitors (SSRIs), reduce anxiety-related behaviors in the EPM test [18]. The interpretation of the FST results, namely the extrapolation from motor activity to mood, should be performed with caution, especially when assuming corresponding mental states in humans from the behavioral responses of rodents during morphine withdrawal. The immobile behavior of rodents in relation to unavoidable and inescapable stress has been hypothesized to indicate depressive disorders associated with mental despair in humans [1]. In the present study, the finding that the WG administration significantly decreased the time spent immobile indicates that WG alleviated depression-like behavior associated with morphine withdrawal.

Increased activity of CRF systems in the brain is involved in behavioral and physiological manifestations of drug withdrawal and in relapses to drug-taking behavior in both animal subjects and human clinical populations [23]. The dysregulation of the CRF systems in the hypothalamus is important in the regulation of anxiety and depression during morphine withdrawal [16]. Our data suggest that the hypothalamic CRF system is potently activated after morphine administration, and this activation might be responsible for inducing anxiety and depression related to morphine withdrawal [14]. In accordance with this observation, the CRF expression in the PVN of the hypothalamus in the MOR group was higher than that in the SAL group. We have shown that WG administration significantly decreased the CRF-like immunoreactivity in the PVN of the hypothalamus. These results suggested that the anxiolytic effect of WG during morphine withdrawal might be closely related to modulation of the CRF system in the hypothalamus. Furthermore, the modulation of the CRF system, typically observed in anxiety and depression related to morphine withdrawal, might be due to activation of the HPA axis [7]. This theory has been supported by some studies in which alteration of HPA axis activity occurred by repeated morphine administration and thus affected behavioral activity and CRF expression via a hypothalamic mechanism [3, 7]. Thus, it could be suggested that the WG appeared to normalize CRF system balance in the hypothalamus by influencing the HPA axis, resulting in the increased negative feedback.

NPY is widely distributed throughout the CNS and is one of the most highly conserved peptides in evolution. Different concentrations of NPY were detected throughout the limbic system, which implied that it is closely related with the modulation of emotional processing and consequently the pathogenesis of depression disorders [12]. Several studies have consistently elucidated that NPY plays a role in depression and in the compensatory mechanisms of antidepressants [24]. In addition, previous studies have shown that NPY levels are correlated with anxiety in animal models of depression, suggesting a possible link between low-level expression of NPY and predisposition to anxiety- or stress-induced depression [10]. Taken together, the decreased expression of NPY in the PVN was elicited by repeated treatment of morphine and subsequent withdrawal [12]. Some additional evidence has supported the hypothesis that enhanced CRF and reduced NPY in the hypothalamus can be related to enhanced anxiety and depression in rats previously exposed to morphine [6]. We propose, therefore, that the beneficial effects of WG are closely related to an increase in NPY expression in the hypothalamus.

WG has a long history of use in drug abuse therapy. WG is a well-known traditional medicine that has long been included in medical prescriptions for treating mental dysfunctions involving drug abuse. Although typical prescriptions including WG are known to be clinically effective for alleviating psychostimulant-induced withdrawal symptoms, the underlying mechanisms of WG in those therapies have not been investigated. The major components of WG are ginsenosides, which have a hydrophobic steroid-like four-ring structure with sugar-attached moieties [13], and their concentrations are generally 2 to 6 times higher than those of field-cultivated ginseng [9]. It was also observed that all ginsenosides analyzed in many studies were present at higher levels in WG than in PG [9]. In particular, the concentrations of Rb1 and Rg1 in WG were 4 to 6 times higher, as compared with PG [9]. These findings indicated that WG might be more effective than PG in
preventing patients with drug addiction from relapsing into drug-seeking while trying to quit, by relieving some of the discomfort of morphine withdrawal symptoms such as anxiety and depression.

In summary, our results demonstrated that WG extract reduced the anxiety- and depression-like symptoms strongly associated with morphine discontinuation, probably by modulating CRF and NPY expressions in the hypothalamus. These results suggest that WG might be an effective agent for curing anxiety and depression disorders, and CRF and NPY might be a biological target or a mechanistic rationale for developing alternative medications in the treatment of anxiety and depression.

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References


