



Valeriana officinalis root extracts have potent anxiolytic effects in laboratory rats

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ABSTRACT

Valerian root (*Valeriana officinalis*) is a popular and widely available herbal supplement, primarily used to treat insomnia and anxiety. Until recently, its mechanism of action has remained unknown. Neurobiological research has begun to show that the herb, with its active valerenic acid, interacts with the GABA_A-ergic system, a mechanism of action similar to the benzodiazepine drugs. This series of experiments sought to corroborate these findings with behavioral measures, compare them to the benzodiazepine diazepam, and to analyze the chemical composition of *Valeriana officinalis*. Rats were administered either ethanol (1 ml/kg), diazepam (1 mg/kg), valerian root extract (3 ml/kg), valerenic acid (3 mg/kg), or a solution of valerenic acid and exogenous GABA (75 µg/kg and 3.6 µg/kg, respectively) and assessed for the number of entries and time spent on the open arms of an elevated plus maze. Results showed that there was a significant reduction in anxious behavior when valerian extract or valerenic acid exposed subjects were compared to the ethanol control group. The evidence supports *Valeriana officinalis* as a potential alternative to the traditional anxiolytics as measured by the elevated plus maze.

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Introduction

Anxiety disorders, including generalized anxiety disorder (GAD), panic disorder, post-traumatic stress disorder (PTSD), and obsessive compulsive disorder (OCD), are the most prevalent behavioral disorders in the United States, affecting 17.2% of the population (Somers et al. 2006). The use of herbal supplements to treat anxiety and insomnia has been increasing and the mechanisms of action of several are being elucidated.

Valerian, derived from the *Valeriana officinalis* plant, is one of the most popular herbal supplements for the treatment of anxiety and insomnia. In spite of its large popularity, scientific research on the efficacy of valerian as an anxiolytic is relatively sparse. Of the literature available, the emphasis has been on its activity on γ -amino butyric acid (GABA) neurons within the central nervous system. For example, Awad et al. (2007) found that an ethanolic extract of *Valeriana officinalis* prompted increased brain GABA levels and neurotransmission by stimulating glutamic acid decarboxylase (GAD) in rat brains, as measured by an *in vitro* enzyme assay. Further, extracts from valerian root facilitated the inhibition of GABA transaminase activity, the enzyme responsible

for breaking down GABA, also measured by an *in vitro* enzyme assay.

While it has been demonstrated that valerian has anxiolytic properties in rodents (Hattesoehl et al. 2008) and sedative properties in humans (Donath et al. 2000), it has been uncertain which active components of valerian produced anxiety reduction. Hendriks and colleagues (1985) implicated valerenic acid, isolated from valerian root, as it produced barbiturate-like effects on performance tests with mice.

Studies have begun to show that the neural action of valerenic acid involves GABA systems of the brain, and to a lesser extent, the serotonergic system (Khom et al. 2007; Dietz et al. 2005). It appears that valerenic acid interacts with GABA_A neurons similarly to action of the benzodiazepines, by binding to specific subunits on the GABA_A receptor complex. Stimulation of GABA_A receptors directly opens chloride channels, thereby producing neural inhibition.

Khom et al. (2007) expressed thirteen different subunits of GABA_A receptors in *Xenopus* oocytes. Using the two-microelectrode voltage-clamp technique, they found that only the chloride channels composed of β_2 and β_3 subunits were stimulated by valerenic acid. Neither the γ subunit, which is usually stimulated by the benzodiazepines, nor the α subunits were stimulated by valerenic acid. The β_2 subunit effect was greatly reduced following a single amino acid point mutation of β_{2N265S} . This

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single amino acid point mutation is also known to inhibit the action of loreclezole, a sedative and anticonvulsant (Wingrove et al. 1994). Further, a single amino acid point mutation of β_{1S290} of the β_1 subunit, which is otherwise not sensitive to valerianic acid, produced a level of sensitivity to valerianic acid comparable to β_2 receptors.

Benke and colleagues (2009) radiolabeled valerianic acid and found that high affinity binding sites for valerianic acid were located on GABA_A receptors. Ligands for the binding sites of GABA, benzodiazepines, barbiturates, picrotoxinin and loreclezole did not affect the binding of valerianic acid. However, the anti-inflammatory mefenamic acid, which is structurally similar to loreclezole, did inhibit valerianic acid binding, which suggests that valerianic acid may bind to an as yet unrecognized site that is allosterically linked to anesthetics and mefenamic acid. Corroborating the findings of Khom et al. (2007), single point mutation of β_{2N265M} of the β_2 subunit of the GABA_A receptor impaired the binding and response of valerianic acid. Single point mutation of β_{3N265M} of the β_3 subunit impaired the binding and behavioral effects of valerianic acid.

While valerian acts mainly through GABA interactions its effects may be further mediated by its ability to enhance the effects of adenosine (Müller et al. 2002). Müller and colleagues conducted an *in vitro* radioligand binding assay at A₁ adenosine receptors on rat brain cortical membranes, and at A_{2A} adenosine receptors on rat brain striatal membranes. They found that valerian bound with high affinity to A₁ adenosine receptors – with 15-fold greater potency than A_{2A} receptors – competitively displacing [³H]N⁶-cyclohexyladenosine in a dose-dependent pattern. These mechanisms likely account for the sedative effects of valerian. Benzodiazepines, too, are often used as sedatives, and additionally act by blocking the reuptake of adenosine and permitting its accumulation (Phillis and O'Regan 1988).

In the present study, we analyzed the anxiolytic effect of valerian root extract, valerianic acid, and concentrations of valerianic acid and GABA isolated in our extract using the elevated plus maze. This method has been often used by researchers to test the anxiolytic properties of drugs and is an effective measure of animal anxiety (Pellow et al. 1985). Our objective was to determine the concentrations of valerianic acid and GABA in a valerian root extract potent enough to produce anxiolysis. These effects were further validated by administering valerianic acid and exogenous GABA to animals in relative concentrations equal to that found in the extract.

Materials and Methods

Chemicals and plant material

Diazepam, valerianic acid, GABA, *trans*-cinnamic acid, and methyl 4-amino-3-bromobenzoate were purchased from Sigma-Aldrich (St. Louis, Missouri). Valerian root was obtained from Chromadex (Irvine, California). Acetonitrile, methanol and water used for LC-MS applications were Optima LC-MS grade obtained from Fisher (Hampton, NH), and 99+% formic acid was obtained from Thermo Scientific in 1-mL ampoules (Rockford, IL). Non denatured ethanol used for extractions and as a drug vehicle was 190-proof and obtained locally.

Valerian root extraction

Valerian root was finely ground using a mortar and pestle and resuspended in a 50% v/v solution of ethanol and distilled H₂O (100 mg/6 ml). The ethanolic solution was covered and heated to

65–70 °C for 30 min, allowed to cool, and then filtered using medium porosity filter paper to remove undissolved solutes (as described in U.S. Patent No. 6,913,770,200). The extract solution was reduced from 6 ml to 3 ml during this process due to ethanol evaporation and some absorption by the filter paper. This solution was administered at a dose of 3 ml/kg of body weight.

Preparation of drugs for administration to animals

Diazepam was dissolved in ethanol at a concentration of 1 mg/ml. Valerianic acid for one group of animals was dissolved in ethanol at a concentration of 3 mg/ml. For another group, valerianic acid was dissolved in ethanol at a concentration of 75 µg/ml and combined with GABA that was dissolved in distilled H₂O at a concentration of 3.6 µg/ml. Control animals received 1 ml/kg (about 0.30 ml) of the ethanol vehicle—a dose that did not produce any measurable sedative or anxiolytic effects in pilot studies. All solutions were approximately neutral pH and isotonic.

Instrumentation for chemical analysis

Valerianic acid and GABA in valerian root were quantified separately using LC-MS with multiple reaction monitoring (MRM). The chromatographic separations were performed using a Waters Alliance 2795 HPLC with a Phenomenex Luna RP-C18 column (3 µ, 50 × 3 mm). The autosampler was kept at 4 °C and the column compartment was heated at 40 °C. Flow rates of 0.5 ml/min were maintained throughout all experiments and all injection volumes were 10 µl. Buffer A was water with 0.1% formic acid (vol/vol), and Buffer B was acetonitrile with 0.1% formic acid (vol/vol). Mass spectroscopy was performed using a Waters Quattro Micro triple-quadrupole mass spectrometer in positive electrospray mode. The desolvation gas was nitrogen and the collision gas was argon. Quattro Micro (QM) parameters were as follows: source temperature, 120 °C; desolvation temperature, 350 °C; cone gas flow, 50 l/h; desolvation gas flow, 750 l/h. Other QM settings specific to each analyte are outlined in the next sections. Data were analyzed using MassLynx version 4.1 and QuanLynx software by Waters.

Quantitation of valerianic acid in valerian root extract

A calibration curve was prepared using valerianic acid standards (25, 50, 75, 100, and 150 ng/ml in methanol) all containing the internal standard, *trans*-cinnamic acid (100 ng/ml). Injections were performed in triplicate. Chromatographic conditions for separation of valerianic acid utilized a linear gradient from time = 0 to 2 min (50:50 A/B to 20:80 A/B). From 2 to 3 min conditions were constant (20:80 A/B), and then starting conditions were resumed and held from 3 to 4 min (50:50 A/B). The capillary voltage on the QM was 3700 V. The MRM transition for valerianic acid was 235.15 > 216.99 m/z, with a dwell time of 0.1 s, cone voltage of 20 V, and collision energy of 12 V. The MRM transition for *trans*-cinnamic acid was 148.95 > 130.79 m/z, with a dwell time of 0.1 s, cone voltage of 20 V, and a collision energy of 10 V.

Valerian root extracts from three separate extractions were each diluted 1:200 in methanol and spiked with *trans*-cinnamic acid (for a final concentration of 100 ng/ml) prior to analysis. Each sample was injected in triplicate.

Quantitation of GABA in valerian root extract

A calibration curve was prepared using GABA standards (50, 75, 100, 150, and 200 ng/ml in methanol) all containing the internal

standard, methyl 3-bromo-4-aminobenzoate (100 ng/ml). Injections were performed in triplicate. Chromatographic separation of GABA required initial conditions of 99.5:0.5 A/B, from time = 0 to 1 min. From 1 to 1.5 min solvent ratios were changed to 20:80 A/B, then held constant from 1.5 to 4.5 min, before returning to starting conditions. The capillary voltage on the QM was 2200 V. The MRM transition for GABA was 103.93 > 86.83 m/z, with a dwell time of 0.1 s, cone voltage of 15 V, and collision energy of 10 V. The MRM transition for methyl 3-bromo-4-aminobenzoate was 237.77 > 134.87 m/z, with a dwell time of 0.1 s, cone voltage of 30 V, and a collision energy of 16 V.

Valerian root extracts from three separate extractions were diluted 1:10 in methanol and spiked with methyl 3-bromo-4-aminobenzoate (final concentration of 100 ng/ml) prior to analysis. Each sample was injected in triplicate.

Animals

Fifty female hooded rats aged six to ten months were used. The animals were randomly assigned into five groups of 10 animals. All animals were housed individually in suspended wire mesh cages in a climatically-controlled room (22 °C) maintained on a 12-hour/12-hour light/dark cycle. Experiments were conducted during the 12-hour light phase. Food and water were available *ad libitum*.

Elevated plus maze

The elevated plus maze consisted of two open and two enclosed arms, arranged oppositely to each other. The arm surfaces were 50 cm long and 10 cm wide, and painted black. The walls of the enclosed arms, also painted black, were 30 cm in height. A small LED light was fixed at the end of each enclosed arm. We found that the small light motivated animals to spend more time exploring the open arms of the maze. The maze was elevated to a height of 50 cm above the floor. The procedure was first described by Pellow et al. (1985) as a behavioral model to measure anxiety in rats. The elevated plus maze was situated in a sound-attenuated room under high illumination.

Drug administration

Animals were randomly selected and administered an intra-peritoneal injection of one of the following treatments: ethanol vehicle (1 ml/kg), diazepam (1 mg/kg), valerian root extract (3 ml/kg), valerianic acid (3 mg/kg), or 1 ml/kg valerianic acid and GABA in ethanol and distilled water at concentrations of 75 µg of valerianic acid and 3.6 µg of GABA in 1 ml in 50/50 mixture of distilled H₂O and ethanol. These concentrations resulted in similar ethanol volumes for each group of animals and for a similar concentration of valerianic acid and GABA found in our valerian extract (see below). All animals were immediately returned to their home cages after injections.

Behavioral testing

Thirty minutes after drug administration animals were placed onto the center of the maze facing an enclosed arm. During a five-minute test session, the animals were allowed to freely explore the maze. The number of open arm entries and the time each animal spent on the open and closed arms were recorded by video camera and later analyzed. An open arm entry was defined as all four paws on an open arm. To avoid habituation to the maze each animal was tested only once.

Results and Discussion

Quantitation of valerian root

The linear regression analysis of both the valerianic acid and GABA calibration curves gave high squared correlation coefficients ($r^2 > 0.995$). The average concentration of valerianic acid in three samples of valerian root extract was determined to be 24.2 µg/ml with a standard deviation of 2.1 µg/ml (Table 1, Fig. 1). The average concentration of GABA in the same three samples of valerian root extract was determined to be 1.2 µg/ml with a standard deviation of 0.1 µg/ml (Table 1, Fig. 2).

Elevated plus maze

The results of the elevated maze experiment are presented in Fig. 3 which compares the mean time spent on the open arms of

Table 1
Mean concentrations of valerianic acid and GABA in valerian root extracts.

Valerian root extract sample	Valerianic acid (µg/ml)		GABA (µg/ml)	
	mean ^a (n=3)	%RDS	mean ^b (n=3)	%RDS
1	26.3	1.1	1.37	1.1
2	24.1	2.7	1.25	1.0
3	22.1	3.1	1.08	1.1
	Valerianic acid (µg/ml)		GABA (µg/ml)	
Mean Extracts 1-3	24.2		1.23	
Std Dev Extracts 1-3	2.1		0.15	

^a The equation for the valerianic acid calibration curve was $y = 0.0079x - 0.0156$, $r^2 = 0.995$, where $y = (\text{peak area of valerianic acid}) / (\text{peak area of trans-cinnamic acid})$, and $x = \text{concentration of valerianic acid in ng/ml}$. The µg/ml values for valerianic acid were obtained by multiplying the dilution factor of 200.

^b The equation for the GABA calibration curve was $y = 0.0111x - 0.0483$, $r^2 = 0.998$, where $y = (\text{peak area of GABA}) / (\text{peak area of methyl 4-amino-3-bromobenzoate})$, and $x = \text{concentration of GABA in ng/ml}$. The µg/ml values for GABA were obtained by multiplying the dilution factor of 10.

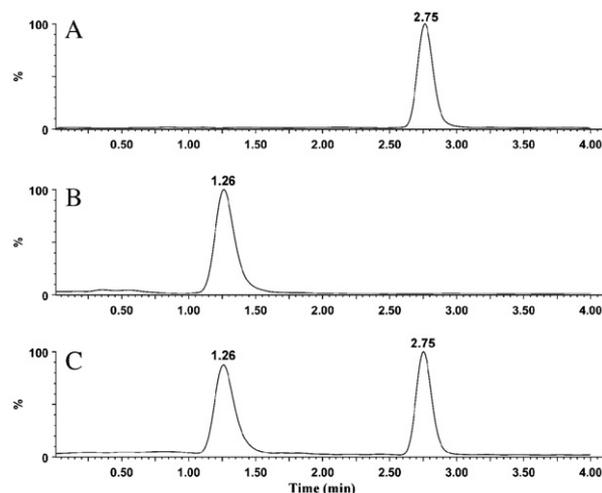


Fig. 1. Valerianic acid in valerian root extract. Extract was diluted by 1:200 for LC-MS analysis. Buffer A = water, 0.1% formic acid. Buffer B = acetonitrile, 0.1% formic acid. A linear gradient was performed from time = 0 to 2 min (50:50 A/B to 20:80 A/B). From 2 to 3 min conditions were constant (20:80 A/B), and then starting conditions were resumed and held from 3 to 4 min (50:50 A/B). Panel A: Valerianic acid MRM transition 235.146 > 216.99 m/z (2.75 min). Panel B: *Trans*-cinnamic acid internal standard MRM transition 148.946 > 130.79 m/z (1.26 min). Panel C: ES+ TIC.

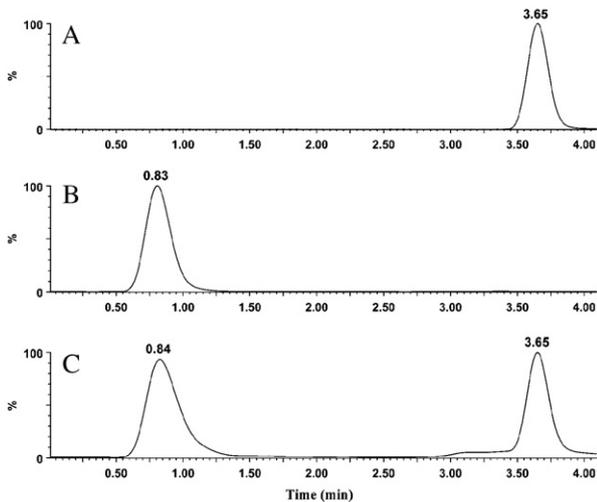


Fig. 2. GABA in valerian root extract. Extract was diluted 1:10 for LC-MS analysis. Buffer A = water, 0.1% formic acid. Buffer B = acetonitrile, 0.1% formic acid. Initial conditions were held from time = 0 to 1 min (99.5:0.5 A/B). From 1 to 1.5 min conditions were changed to 20:80 A/B, then held constant from 1.5 to 4.5 min, before returning to starting conditions (99.5:0.5 A/B). Panel A: Methyl 4-amino-3-bromobenzoate internal standard MRM transition 231.77 > 134.87 m/z (3.65 min). Panel B: GABA MRM transition 103.93 > 86.83 m/z (0.84 min). Panel C: ES+ TIC.

the elevated maze for the five minute test period, 30 minutes following drug treatment. Time spent on the open arms was analyzed using one-way analysis of variance (ANOVA) and Tukey's post-hoc multiple comparisons. There was a significant difference in time spent on the open arms between the treatment groups ($F(4,44) = 4.81, p < 0.005$). The Tukey's post-hoc analysis showed that animals in the drug treatment groups spent significantly greater time on the open arms than the ethanol control group. Within the drug treatment groups, there were no significant differences in anxiolytic effects. As shown in Fig. 3, the anxiolytic diazepam increased time on the open arms almost threefold over ethanol control times (25.1 vs 68.8 sec). This increase in time spent on the open arms cannot be attributed to depressed motor functioning as these animals demonstrated normal motor behavior and coordination throughout testing. In addition, as shown in Fig. 4, the number of open arm walkouts for drug treated animals was significantly higher when compared to the ethanol control group ($F(4,44) = 4.20, p < 0.02$). The number of open arm walkouts between drug treated groups, however, was not significantly different. These results indicate that the anxiolytic effects observed were not confounded by locomotor inhibition.

Pure valerianic acid in the 3 mg/kg dosage produced the highest anxiolytic effect with a mean of 81.0 seconds spent on the open arm, compared to the ethanol control group mean of 25.1 seconds. This result was predicted based on doses of valerianic acid used by Benke et al. (2009) Again, valerianic acid did not appear to disrupt motor behavior or coordination at these doses. Surprisingly, the relatively low dose of valerianic acid when combined with GABA in concentrations equivalent to concentrations in our valerian extract was also quite potent. We presume that this potency reflects an enhanced bioactivity of valerianic acid in the presence of exogenous GABA.

Controversy remains regarding the influx capability of GABA into the brain from the blood stream. Al-Awadi and colleagues (2006) showed that GABA crossed the blood-brain barrier. They compared GABA levels in the brains of hypertensive and nonhypertensive rats following intravenous administration of exogenous GABA into the femoral artery. Dosages ranged from

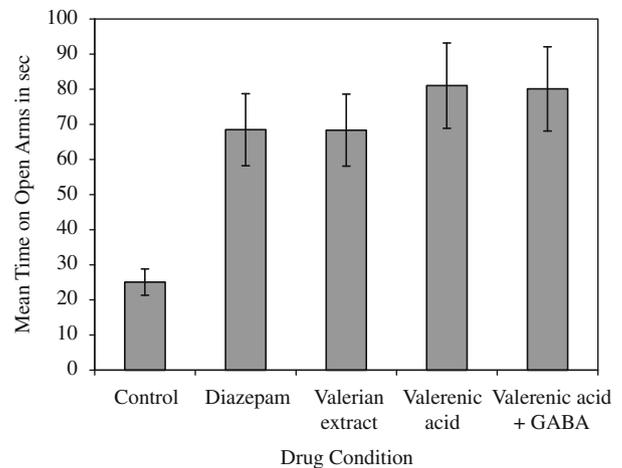


Fig. 3. Elevated plus maze. Mean time (+SE) in sec spent on the open arms following the administration of the ethanol control (1 ml/kg), diazepam (1 mg/kg), valerian root extract (3 ml/kg), valerianic acid (3 mg/kg), or valerianic acid with GABA (25 µg/ml and 1.2 µg/ml), respectively.

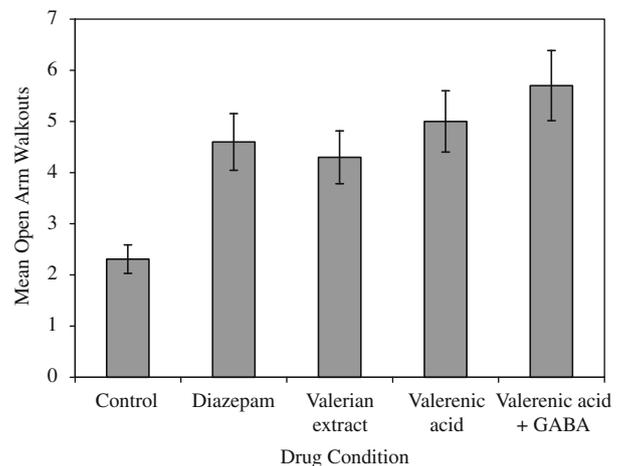


Fig. 4. Open arm entries. Mean number (+SE) of open arm entries defined as all four paws on an open arm following the administration of the ethanol control (1 ml/kg), diazepam (1 mg/kg), valerian root extract (3 ml/kg), valerianic acid (3 mg/kg), or valerianic acid with GABA (25 µg/ml and 1.2 µg/ml), respectively.

4 µg/kg (a concentration only slight greater than our 3.6 µg/kg) to 5 mg/kg. High performance liquid chromatography (HPLC) was used to measure concentrations of GABA in various brain regions, as well as cerebrospinal fluid (CSF). Levels of GABA measured in the CSF as well as several brain structures increased in a dose-dependent pattern. While brain uptake was greater in hypertensive rats, it occurred in the nonhypertensive rats as well (~0.3 ml/kg).

Al-Awadi et al. (2006) waited only five minutes following administration of GABA before the assays were performed, which may not have allowed GABA the time to reach even higher concentrations in the brain. Further, they did not measure GABA levels in the amygdala, a structure that is central to the regulation of anxiety, and with which benzodiazepines interact to produce their anxiolytic effect. Though the GABA influx was small, many drugs that interact directly with receptors are capable of producing powerful behavioral effects in small amounts. Until further experiments have excluded the possibility that GABA crosses the blood brain barrier and produces behavioral effects, this remains a plausible explanation. Moreover, the synergistic effects of valerianic acid and GABA in combination may facilitate

transport across the blood brain barrier, and produce a unique molecular action in the brain as yet unknown.

It is also possible that the exogenous GABA in our sample produced periphery anxiolytic effects. Metzeler and colleagues (2004) used methods of immunocytochemistry, immunoblots, and reverse transcription polymerase chain reaction (RT-PCR) to demonstrate the presence of local GABA production by the decarboxylation of glutamic acid in human and rat adrenocortical cells. Immunostaining and whole-cell patch clamp techniques confirmed the presence and functionality of GABA_B receptors. They suggest that the synthesis and reception of GABA on the adrenal cortex could influence the release of glucocorticoids in a paracrine or autocrine signalling manner. The regulation of glucocorticoid release may indirectly mediate some of the anxiolytic effects of the GABA present in valerian root.

Our findings have strong implications for the general use of valerian root as an anxiolytic. One of several questions prompted by these implications relates to valerian's ability to treat anxiety in humans compared to traditional anxiolytics, and whether cross-tolerance to valerian would occur in those who have become tolerant to the effects of the benzodiazepines. Further, it is typically true of the elevated plus maze method that there is considerable variability in the behavior of the animals. This was true of our results. To avoid such variability in future behavioral work, methodology should involve a more precise measure of anxiolysis, such as an avoidance-escape task.

In conclusion, there is growing reason to believe that valerian root may be an effective alternative to the traditional anxiolytics, which often produce such aversive side effects as nausea, tremor, and addiction (Stewart and Westra 2002). The physiological mechanisms of valerian action on the central nervous system are becoming better established, as are the resultant behavioral effects. We have determined the relative concentrations of the primary constituents of valerian root extract and what quantity of the herb is efficacious. Further we have shown that valerenic acid is the primary anxiolytic component and its effects are enhanced by the presence of GABA. Future research should emphasize methods of greater precision in the behavioral studies.

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