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A cycloartane glycoside derived from *Actaea racemosa* L. modulates $GABA_A$ receptors and induces pronounced sedation in mice

Barbara Strommer, Sophia Khom, Iris Kastenberger, Serhat Sezai Cicek, Hermann Stuppner, Christoph Schwarzer, Steffen Hering

Department of Pharmacology and Toxicology, University of Vienna; Althanstraße 14, A-1090 Wien; Austria (BS, SK, SH)

Department of Pharmacology, Medical University Innsbruck; Peter Mayr Straße 1a, A-6020 Innsbruck; Austria (IK, CS)

Institute of Pharmacy/Pharmacognosy, Center for Molecular Biosciences Innsbruck (CMBI), University of Innsbruck, Innrain 52c, A-6020 Innsbruck, Austria (SSC, HS)

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Running title: $GABA_A$ receptor modulation by Ac-SM

To whom correspondence should be addressed:

ao. Prof. Dr. Mag. Christoph Schwarzer

Department of Pharmacology, Innsbruck Medical University

Peter-Mayr-Str. 1a, A-6020 Innsbruck

Email: schwarzer.christoph@i-med.ac.at

Phone: +43 512 9003 71205

Fax: + 43 512 9003 73200

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Ac-SM (23-*O*-Acetylshengmanol 3-*O*-β-D-xylopyranoside)

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Abstract

23-*O*-acetylshengmanol-3-*O*-β-d-xylopyranoside (Ac-SM) isolated from *Actaea racemosa* L. - an herbal remedy for the treatment of mild menopausal disorders- has been recently identified as a novel efficacious modulator of γ-aminobutyric acid (GABA) type A ($GABA_A$) receptors composed of α_1-, β_2- and γ_{2S}-subunits. In the present study, we analyzed a potential subunit-selective modulation of GABA-induced chloride currents (I_{GABA} EC_{3-8}) through nine $GABA_A$ receptor isoforms expressed in *Xenopus laevis* oocytes by Ac-SM with 2-microelectrode-voltage-clamp and behavioral effects 30 min after intraperitoneal application in a mouse model. Efficacy of I_{GABA} enhancement by Ac-SM displayed a mild α-subunit-dependence with $\alpha_2\beta_2\gamma_{2S}$ (maximal I_{GABA} potentiation E_{max}=1454±97%) and $\alpha_5\beta_2\gamma_{2S}$ (E_{max}=1408±87%) receptors being most efficaciously modulated, followed by slightly weaker I_{GABA} enhancement through $\alpha_1\beta_2\gamma_{2S}$ (E_{max}=1187±166%), $\alpha_3\beta_2\gamma_{2S}$ (E_{max}=1174±218%) and $\alpha_6\beta_2\gamma_{2S}$ (E_{max}=1171±274%) receptors and less pronounced effects on receptors composed of $\alpha_4\beta_2\gamma_{2S}$ (E_{max}=752±53%) subunits, while potency was not affected by the subunit composition (EC50 values ranging from $\alpha_1\beta_2\gamma_{2S}$=35.4±12.3μM to $\alpha_5\beta_2\gamma_{2S}$=50.9±11.8μM). Replacing β_2- by β_1- or β_3-subunits as well as omitting the γ_{2S}-subunit affected neither efficacy nor potency of I_{GABA} enhancement by Ac-SM. Ac-SM shifted the GABA-concentration-response-curve towards higher GABA sensitivity (about 3-fold) and significantly increased the maximal GABA response by 44±13% indicating pharmacological profile distinct from a pure allosteric $GABA_A$ receptors modulator. In mice, Ac-SM significantly reduced anxiety-related behavior in the elevated-plus-maze-test at a dose of 0.6mg/kg, total ambulation in the open-field-test at doses ≥6mg/kg, stress-induced hyperthermia at doses ≥0.6mg/kg,

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and significantly elevated seizure threshold at doses ≥20mg/kg bodyweight. High efficacy and long biological half-life of Ac-SM suggest that potential cumulative sedative side effects upon repetitive intake of *Actaea racemosa* L. preparations might not be negligible.

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Introduction

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γ-aminobutyric acid (GABA) mediates fast inhibitory neurotransmission in the mammalian brain by activating synaptic and extrasynaptic GABA type A ($GABA_A$) receptors. $GABA_A$ receptors are composed of five subunits that form a chloride conducting ion pore (Macdonald and Olsen, 1994; Sieghart, 1995; Sieghart and Sperk, 2002; Sigel and Steinmann, 2012). 19 different $GABA_A$ receptor subunits have been identified in the human genome (Simon *et al.*, 2004) allowing theoretically the formation of approximately 800 subunit combinations. However, so far the existence of only 11 native $GABA_A$ receptors subtypes has been confirmed (Olsen and Sieghart, 2008). There is consensus that the mainly expressed $GABA_A$ receptor isoform consists of α_1, β_2, and γ_2 subunits (Olsen and Sieghart, 2008) with a commonly accepted 2α:2β:1γ subunit stoichiometry (Tretter *et al.*, 1997; Baumann *et al.*, 2001, 2002). The subunit combination determines the pharmacological properties of $GABA_A$ receptors (Olsen and Sieghart, 2009). $GABA_A$ receptors play a major role in controlling the excitability of the mammalian brain and are thus involved in regulating amongst others sleep, vigilance, mood and emotions (Möhler *et al.*, 2005; Möhler, 2006). $GABA_A$ receptors are modulated by structurally diverse compounds including clinically applied drugs such as benzodiazepines, barbiturates, neurosteroids, anaesthetics as well as a large number of natural products including flavonoids, terpenoids or polyacetlyenes (for review see Johnston *et al.*, 2006; Olsen and Sieghart, 2009; Hanrahan *et al.*, 2011; Nilsson and Sterner, 2011). Recently, we have identified four cycloartane glycosides (actein, cimigenol 3-*O*-β-D-xylopyranoside, 25-*O*-acetylcimigenol 3-*O*-α-L-arabinopyranoside and 23-*O*-acetylshengmanol 3-*O*-β-D-xylopyranoside) as novel efficacious $GABA_A$ modulators isolated from *Actaea racemosa L.* While I_{GABA} enhancement by actein,

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cimigenol 3-*O*-β-D-xylopyranoside and 25-*O*-acetylcimigenol 3-*O*-α-L-arabinopyranoside averaged at about 300%, a more than 5-fold stronger I_{GABA} potentiation was observed for the cycloartane glycoside 23-*O*-acetylshengmanol 3-*O*-β-D-xylopyranoside (Ac-SM) (Cicek *et al.*, 2010).

The aim of the present study was to obtain deeper insights into the molecular mechanism of Ac-SM action by analyzing a potential subunit-dependent modulation of nine different $GABA_A$ receptor subtypes expressed in *Xenopus laevis* oocytes. In addition, potential anxiolytic, stress-reducing, anticonvulsant effects as well as changes in locomotor activity upon application of Ac-SM were studied in mice to investigate the potential effects of this compound on mood, sedation and seizure threshold.

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Materials and Methods

Animals

All procedures involving animals were approved by the Austrian Animal Experimentation Ethics Board in compliance with the European convention (Directive 2010/63/EU) for the protection of vertebrate animals used for experimental and other scientific purposes ETS no.: 123. Every effort was taken to minimize the number of animals used.

Chemicals

Ac-SM (Fig. 1) was isolated from *Actaea racemosa* L. as previously described (Cicek *et al.*, 2010). Stock solutions (100mM for *in vitro* experiments and 1mg/10µl for *in vivo* experiments, respectively) were prepared in dimethylsulfoxide (DMSO). All chemicals were obtained from Sigma Aldrich (Vienna, Austria).

Due to low solubility in the bath solution Ac-SM was used only up to a concentration of 300µM in *in vitro* experiments. Equal amounts of DMSO were present in control and compound-containing solutions. The maximum DMSO concentration in the bath (0.3%) did not affect I_{GABA} (Khom *et al.*, 2006).

For *in vivo* experiments, working concentrations were adjusted by dilution with 0.9% sodium chloride; the final concentration of DMSO was fixed to 10% including control solutions. To enhance solubility of the compound Tween 80 (3% final concentration) was added to all solutions. pH was adjusted to 7.2-7.4 with 1M sodium hydroxide. All solutions were freshly prepared every day prior to experiments.

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Expression and functional characterization of $GABA_A$ receptors

Preparation of stage V-VI oocytes from *Xenopus laevis* (NASCO, Fort Atkinson, USA), synthesis of capped off run-off poly(A^+) rat cRNA transcripts from linearized cDNA templates (pCMV vector) was performed as described (Khom *et al.*, 2006). Briefly, female *Xenopus laevis* were anaesthetized by exposing them for 15min to a 0.2% solution of MS-222 (methane sulfonate salt of 3-aminobenzoic acid ethyl ester) before surgically removing parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2mg/ml collagenase (Type 1A). Oocytes were stored at 18°C in ND96 solution (Methfessel *et al.*, 1986). One day after isolation, the oocytes were injected with about 10-50nl of diethyl pyrocarbonate (DEPC) - treated water containing the different rat cRNAs (150 - 3000ng/µl /subunit). To ensure expression of the γ_{2S}-subunit in the case of $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, $\alpha_5\beta_2\gamma_{2S}$, $\alpha_6\beta_2\gamma_{2S}$ and $\alpha_1\beta_3\gamma_{2S}$ receptors, cRNAs were mixed in a ratio of 1:1:10 (Boileau *et al.*, 2002) except for $\alpha_4\beta_2\gamma_{2S}$ receptors a ratio 3:1:5 was used. For $\alpha_1\beta_2$ receptors, cRNAs were mixed in a ratio 1:1. cRNAs for $\alpha_1\beta_1\gamma_{2S}$ channels were injected in a ratio of 3:1:10 to avoid formation of β_1-homooligomeric $GABA_A$ receptors (Krishek *et al.*, 1996). The amount of cRNA was determined by means of a NanoDrop ND-1000 (Kisker-biotech, Steinfurt, Germany).

Electrophysiological experiments were done using the two-microelectrode-voltage-clamp-technique at a holding potential of -70 mV making use of a TURBO TEC 01C amplifier (npi electronic, Tamm, Germany) and an Axon Digidata 1322A interface (Molecular Devices, Sunnyvale, CA). Data acquisition was carried out using pCLAMP v.9.2 (Molecular Devices, Sunnyvale, CA). The bath solution contained 90mM NaCl,

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1mM KCl, 1mM $MgCl_2 \cdot 6H_2O$, 1mM $CaCl_2$ and 5mM HEPES (pH 7.4). Microelectrodes were filled with 2M KCl and had resistances between 1 and 3 MΩ (Khom *et al.*, 2006).

Perfusion system

GABA and Ac-SM were applied by means of fast perfusion system (for details see Baburin *et al.*, 2006). Drug or control solutions were applied by means of a TECAN Miniprep 60 permitting automation of the experiments. To elicit I_{GABA} the chamber was perfused with 120 µl of GABA-containing solution at volume rate between 300 and 1000 µl/s. The I_{GABA} rise time ranged between 100 and 250ms (Khom *et al.*, 2006). To account for possible slow recovery from increasing levels of desensitization in the presence of high GABA or compound concentrations, the duration of washout periods was extended stepwise, i.e. 1.5 min (GABA EC_{3-8}) to 3min (co-application of GABA EC_{3-8} in the presence ≤ 1µM Ac-SM;) to 5-10min (co-application of GABA EC_{3-8} in the presence of ≤ 10 µM compound) to 15-20min (co-application of GABA EC_{3-8} and ≤ 100µM Ac-SM) to 30min (GABA EC_{3-8} in the presence of 300 µM Ac-SM). The duration of washout periods in experiments analyzing the effect of Ac-SM (300 µM) at different GABA concentrations was fixed to 30min. Potential run-down or run-up effects were ruled out by application of GABA control at the end of each experiment. Oocytes with maximal current amplitudes >3 µA were discarded to exclude voltage-clamp errors (Khom *et al.*, 2006).

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Analyzing concentration-response curves

Stimulation of chloride currents by modulators of the $GABA_A$ receptor was measured at a GABA concentration eliciting between 3 and 8% of the maximal current amplitude (EC_{3-8}). The EC_{3-8} was determined at the beginning of the experiment for each oocyte by application of 1mM GABA followed by submaximal GABA concentrations.

Enhancement of the chloride current was defined as $(I_{(GABA+Comp)}/I_{GABA}) - 1$, where $I_{(GABA+Comp)}$ is the current response in the presence of Ac-SM and I_{GABA} is the control GABA current. To measure the sensitivity of the $GABA_A$ receptor for a given compound, it was applied for an equilibration period of 1min before applying GABA (EC_{3-8}). Concentration-response curves were generated and the data were fitted by non-linear regression analysis using Origin software (OriginLab Corporation, USA). Data were fitted to the equation: $\frac{1}{1+\left(\frac{EC_{50}}{[Comp]}\right)^{n_H}}$, where n_H is the Hill coefficient. Each data point represents the mean±SEM from at least 3 oocytes and ≥ 2 oocyte batches.

Animals

Male mice (C57BL/6N) were obtained from Charles River Laboratories (Sulzfeld, Germany). For maintenance, mice were group-housed (maximum 5 mice per type IIL cage) with free access to food and water. At least 24h before the commencement of experiments, mice were transferred to the testing facility, continuing *ad libitum* access to food and water. The temperature in the holding and testing facilities was 22±2°C; the humidity was 40-60%; a 12h light-dark cycle was in operation (lights on from 07.00 to 19.00). Male mice aged 3-6 months were tested.

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Intraperitoneal (i.p.) injection of control or Ac-SM-containing solutions was usually done 30min before the test, except for home cage (HC) activity analysis (60min before test) and measurement of stress-induced hyperthermia (3h before test) to reduce the impact of stress on the analysis. Application of the solvent alone did not influence animal behavior as compared to saline-treated controls. All doses are indicated as mg/kg bodyweight of the animal.

Open-field-test

Exploration of a novel environment was tested over 10min in a 50x50cm box build from gray PVC equipped with infra-red beams. Illumination intensity was set to 150 lux in the center. Animals' explorative behavior was analyzed using ActiMot-2 equipment and software (TSE-systems, Bad Homburg, Germany). Arenas were subdivided into 3 fields: border (up to 8cm from wall), center (20x20cm, i.e. 16% of total area), and intermediate area according to the recommendations of EMPRESS (European Mouse Phenotyping Resource of Standardised Screens; http://empress.har.mrc.ac.uk).

Home Cage Activity (HC)

For HC activity mice were singly placed into a type IIIL cage with free access to food and water, enriched with a plastic tube. Movement of mice was monitored through an infra-red detection system (InfraMot, TSE-systems, Bad Homburg, Germany). HC behavior analysis was started at 5pm and ran for 72h. Arbitrary activity counts (AAC) were cumulated in 60 minutes intervals.

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Elevated-plus-maze (EPM)-test

The animal's behavior was tested over 5min on an elevated plus maze 1m above ground. The EPM consisted of 2 closed arms (walls 20cm in height) and 2 open arms, facing each other. The test instrument was built from gray PVC, each arm 50x5cm in size. Illumination intensity was set to 180 lux at the intersection of the arms (defined as the neutral field). Animals were placed into the center, facing an open arm (OA). Analysis of open arm entries time on open arms and distance on the open arms was automatically done with Video-Mot 2 equipment and software (Wittmann *et al.*, 2009).

S*tress-induced-hyperthermia-test*

Stress-induced hyperthermia (SIH) was assessed as described previously (Wittmann *et al.*, 2009). In short: a temperature probe, lubricated with glycerol, was inserted into the rectum of the mouse to a depth of up to 2cm to measure basal temperature T1. The temperature probe remained in the animal till a stable temperature was reached (maximum 10s). Measurement of T1 served also as the handling stressor. After 15min the measurement was repeated (T2) and the rise in temperature (delta T) was considered as stress-induced hyperthermia (Olivier *et al.*, 2003).

Seizure threshold

Seizure threshold was determined by pentylenetetrazole (PTZ) tail-vein infusion on freely moving animals at a rate of 100µl/min (10 mg/ml PTZ in saline, pH=7.4). Infusion was stopped when animals displayed generalized clonic seizures. Animals were immediately

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killed by cervical displacement after onset of seizures. The seizure threshold dose was calculated from the infused volume in relation to body weight.

Statistical analysis

Statistical significance was calculated by one-way ANOVA and where appropriate by two-way ANOVA (followed by a post-hoc mean comparison with Bonferroni; GraphPad; La Jolla, CA; USA). *P*-values of <0.05 were accepted as statistically significant. All data are given as mean±SEM.

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Results

In the present study we demonstrate a comparable potency and efficacy of I_{GABA} modulation by Ac-SM (for structural formulae, see Fig.1) on nine different $GABA_A$ receptor subtypes. I_{GABA} modulation was independent of the presence of a γ_{2S}-subunit. Ac-SM induces anxiolytic, stress-reducing, sedative and anticonvulsant effects.

I_{GABA} enhancement by Ac-SM does not require the presence of a γ_{2S}-subunit

Ac-SM efficaciously potentiates I_{GABA} through $GABA_A$ receptors composed of $\alpha_1\beta_2\gamma_{2S}$ subunits (Cicek *et al.*, 2010). As illustrated in Fig.2A, Ac-SM, however, also modulated $GABA_A$ receptors lacking a γ_{2S}-subunit with similar efficacy and potency ($E_{max}(\alpha_1\beta_2\gamma_{2S})$: 1187±166% and $EC_{50}(\alpha_1\beta_2\gamma_{2S})$: 47.1±20.9µM vs. $E_{max}(\alpha_1\beta_2)$: 1277±243% and $EC_{50}(\alpha_1\beta_2\gamma_{2S})$: 45.0±11.4µM; see also Table 1).

Ac-SM enhances I_{GABA} irrespective of the $GABA_A$ subunit composition

In order to investigate a possible α-subunit-dependent I_{GABA} enhancement, the α_1-subunit was substituted by α_2-, α_3-, α_4-, α_5- and α_6-, respectively and expressed in combination with β_2- and γ_{2S}-subunits (Fig.2B, Table 1). The highest efficacy was observed for receptors containing either α_2- or α_5-subunits, with a maximal I_{GABA} potentiation of $E_{max}(\alpha_2\beta_2\gamma_{2S})$: 1454±97% (n=7) and $E_{max}(\alpha_5\beta_2\gamma_{2S})$: 1408±87% (n=3), respectively (Table 1), followed by receptors comprising either α_1- ($E_{max}(\alpha_1\beta_2\gamma_{2S})$: 1187±166%; n=6), α_3- ($E_{max}(\alpha_3\beta_2\gamma_{2S})$: 1174±218%; n=4) or α_6-subunits ($E_{max}(\alpha_6\beta_2\gamma_{2S})$: 1171±274%; n=3). Ac-SM, however, displayed a lower efficacy on $GABA_A$ receptors comprising α_4-subunits

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compared to $\alpha_1\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, $\alpha_6\beta_2\gamma_{2S}$ ($p<0.05$), $\alpha_2\beta_2\gamma_{2S}$ and $\alpha_5\beta_2\gamma_{2S}$ ($p<0.001$) receptors ($E_{max}(\alpha_4\beta_2\gamma_{2S})$752±53%; n=3; $F_{5,20}$=9.185; Table1).

In contrast to this moderate α-subunit-dependence, replacing the β_2-subunit in $\alpha_1\beta_2\gamma_{2S}$ receptors by either β_1- or β_3-subunits (Fig.2C), did not affect the strength of I_{GABA} enhancement by Ac-SM ($E_{max}(\alpha_1\beta_1\gamma_{2S})$: 1069±214%,n=6 vs. $E_{max}(\alpha_1\beta_2\gamma_{2S})$: 1187±166%, n=6 vs. $E_{max}(\alpha_1\beta_3\gamma_{2S})$: 1247±222%,n=4).

Comparison of the potencies of the respective $GABA_A$ receptor isoforms revealed no significant differences, with EC_{50} values ranging from 35.4±12.3μM ($\alpha_1\beta_3\gamma_{2S}$) to 50.9±11.8μM ($\alpha_5\beta_2\gamma_{2S}$) (see Table 1). Typical current traces for concentration-dependent I_{GABA} enhancement by Ac-SM are illustrated in Fig.2D.

Ac-SM shifts the GABA-concentration-response curves and enhances $I_{GABA\text{-}max}$

To gain further insight into the mechanism of I_{GABA} modulation by Ac-SM, we compared GABA-concentration-response curves in the presence and absence of Ac-SM on $\alpha_1\beta_3$ channels. Ac-SM was applied at a saturating concentration of 300μM. At this concentration, Ac-SM shifted the concentration-effect curve towards significantly lower GABA concentrations ($EC_{50(GABA)}$: 3.7±0.4μM vs. $EC_{50(GABA+Ac\text{-}SM)}$: 1.2±0.2μM, $p<0.01$) and also increased the maximal GABA response by 44±13% (n=3, see Fig.3). Ac-SM at 300μM induced only small currents in the absence of GABA (not exceeding 1% of maximal I_{GABA} induced by 1mM GABA) indicating modulatory effects (see also (Cicek *et al.*, 2010)).

Ac-SM dose-dependently reduces locomotor activity

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In order to get first insight into the *in vivo* effects of Ac-SM, the explorative behavior of male C57BL/6N mice was studied 30min after intraperitoneal application in the open-field-test (OF). As illustrated in Fig.4A, no changes in total ambulation were observed upon application of Ac-SM at doses of ≤2mg/kg. Reduced ambulation compared to solvent-treated controls started at doses ≥6mg/kg reaching a maximum at a dose of 60mg/kg by reducing the total distance by more than 60% (Control: 38.0±1.2m vs. Ac-SM 60mg/kg: 14.7±3.1m; $F_{(6,84)}$=19.597, p<0.01). Time spent in the center of the OF did not significantly differ between compound-treated (doses ≤20mg/kg) and control group. Only mice treated with Ac-SM at a dose of 60mg/kg spent significantly more time in the center area of the OF (Control: 57.7±6.1s vs. Ac-SM 60mg/kg: 212.5±101.6s; $F_{(6,84)}$=2.815, p<0.05; see Fig.4B) reflecting primarly the strong suppression of motor activity. The number of entries into the center area mostly paralleled the time spent in this compartment. However, mice treated with 0.2mg/kg Ac-SM tended to visit the center area more frequently compared to the control group (Control: 28.2±2.1 vs. Ac-SM 0.6mg/kg: 33.1±4.1). In line with reduced ambulation also the number of center entries significantly dropped at a dose of 60mg/kg (Control: 28.2±2.1 vs. Ac-SM 60mg/kg: 5.9±1.6; $F_{(6,84)}$=8.828, p<0.01; see Fig.4C). Control mice and Ac-SM-treated mice covered a comparable percentage of total distances in the center area (see Fig.4D). Higher doses than 60mg/kg were not tested due to limited solubility of the compound.

To investigate the duration of *in vivo* Ac-SM effects, we investigated locomotor activity of mice treated with a dose inducing marked reduction of ambulation. Home cage activity analysis (Fig. 5) revealed a long lasting reduction of motor activity at a dose of 20mg/kg for at least 6-12h. No rebound effect on the subsequent time periods was measured (2-

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way ANOVA, drug effect: $F_{(1,670)}$=16.81, p<0.0001; time effect: $F_{(66,670)}$=5.77, p<0.0001).

Ac-SM paradigm-dependently reduces anxiety-related behavior and stress-induced hyperthermia

Ac-SM slightly reduced motor activity at doses >2mg/kg in the OF compared to vehicle (see Fig.4A). Thus, to assess changes in anxiety-related behavior, the effect of lower doses was analyzed in the elevated-plus-maze-test: as illustrated in Fig.6A, Ac-SM-treated mice (0.6mg/kg) visited the open arms (OA) of the EPM more frequently (control: 15.0±1.6% of total entries vs. Ac-SM 0.6mg/kg: 28.3±4.5% of total entries; $F_{(2,37)}$=4.667; p<0.05). In addition, a tendency towards more time spent (Fig.6B) and longer distances covered on the OA compared to control animals was observed (Fig.6C), these effects were, however, not statistically significant. Total motor activity was not affected at these doses (data not shown).

Studying potential anxiolytic effects at higher doses was, however, hampered by the concomitantly occurring reduction of motor activity that might easily mask anxiolytic effects, as the behavioral tests (OF and EPM) rely on exploratory behavior. Therefore, we included a locomotion-independent test of a physiological stress response. Activation of the autonomic nervous system in response to stress such as exposure to noise, heat or pain in animals can be measured by changes in body temperature- a process referred to as stress-induced hyperthermia (SIH). SIH is a short lasting body temperature elevation (ΔT) that can be reduced or even ablated by anxiolytic drugs (Vinkers *et al.*, 2008). Basal

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body temperature measured before compound application did not differ between control and compound-treated mice (data not shown).

As shown in Fig.7, body temperature upon stress exposure (i.e. insertion of the temperature probe) was raised in saline-treated control mice by 1.2±0.1°C. 3 hours after application, Ac-SM at doses ≥0.6mg/kg significantly reduced ΔT in a dose-dependent manner (0.6mg/kg: ΔT: 0.6±0.1°C; $F_{(4,41)}$=15.350; p<0.01). The maximal effect was reached at a dose of 6mg/kg (ΔT=0.1±0.1°C; $F_{(4,41)}$=15.350; p<0.01), where SIH was not traceable any more.

Ac-SM increases the seizure threshold upon pentylenetetrazole infusion.

A common feature of most positive allosteric modulators of $GABA_A$ receptors is an increased threshold against pentylenetetrazole-induced seizures (De Deyn and Macdonald, 1989; Rudolph *et al.*, 1999; Gerak and France, 2011; Löscher *et al.*, 2013). Indeed, Ac-SM significantly increased the seizure threshold at doses ≥20mg/kg (Control: 40.4±3.5mg/kg PTZ; vs. Ac-SM 20mg/kg: 58.5±1.7mg/kg PTZ; $F_{(6,23)}$=19.903, p<0.001; see Fig.8).

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Discussion

Black cohosh (*Actaea racemosa L.*) preparations are widely used, mainly to relieve menopausal symptoms, although their efficacy is still controversially discussed (Borrelli and Ernst, 2008; Rees, 2009; Sassarini and Lumsden, 2010; Bedell *et al.*, 2012, 2014; Wuttke *et al.*, 2013). Despite extensive studies on the plants' potential active principles, its mechanism of action is still not entirely understood (Palacio *et al.*, 2009; Wuttke *et al.*, 2013; Bedell *et al.*, 2014). Understanding its molecular mechanism(s) of action is, however, a *conditio sine qua non* for considering potential beneficial and unwanted side effects. The initially suggested estrogenic activity of lipophilic components such as triterpenoids was disproved by later studies (Gaube *et al.*, 2007; Mercado-Feliciano *et al.*, 2012; Hajirahimkhan *et al.*, 2013). There is, however, evidence that *Actaea racemosa L.* preparations interact with distinct neurotransmitter systems in the central nervous system including. μ-opiod receptors, (Rhyu *et al.*, 2006; Reame *et al.*, 2008). serotonin (5-HT)-receptors (subtypes 5-HT_{1A}, 5-HT_{1D} and 5-HT_7 (Burdette *et al.*, 2003) and dopamine subtype 2 (D2) receptors (Jarry *et al.*, 2003). We have previously shown that *Actaea racemosa L.* extracts efficaciously modulate $GABA_A$ receptors and identified four structurally related cycloartane glycosides in *Actaea racemosa L.* extracts as novel class of $GABA_A$ receptor ligands (Cicek *et al.*, 2010). Although the potency of these compounds was apparently rather low, we observed a remarkably high efficacy of I_{GABA} enhancement compared to established $GABA_A$ receptor modulators such as benzodiazepines (e.g. maximal I_{GABA} enhancement by the highly potent benzodiazepine site ligand triazolam $E_{max(\alpha1\beta2\gamma2S)}$=253±12% (data taken from (Khom *et al.*, 2006)) vs. I_{GABA} enhancement by Ac-SM $E_{max(\alpha1\beta2\gamma2S)}$=1187±166 in this study (Sigel and Baur,

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1988)). Here, we analyzed potential subunit-selective effects of the most efficacious compound Ac-SM. Ac-SM positively allosterically modulated I_{GABA} trough nine major $GABA_A$ receptor subtypes with equal potency, but slightly different efficacy (Fig.2A-C). Enhancement of I_{GABA} by Ac-SM was only slightly influenced by the the α-subunit isoform incorporated into the channel, but not by the β-subunit isoform and did not depend on the presence of a γ_{2S}-subunit. Based on these observations, we exclude an interaction of Ac-SM with the benzodiazepine binding site (Sigel and Buhr, 1997; Rudolph *et al.*, 1999; Olsen and Sieghart, 2009; Richter *et al.*, 2012; Sigel and Steinmann, 2012). In addition, as Ac-SM efficaciously modulates a broad variety of $GABA_A$ receptor subtypes/ does not discriminate between various $GABA_A$ receptor subtypes, its profile apparently reminds of $GABA_A$ receptor modulators such as barbiturates or neurosteroids. However, whether Ac-SM also shares a common binding site with these compounds or whether Ac-SM interacts with a novel, yet undefined binding site on $GABA_A$ receptors remains to be exploited in future studies.

Our *in vivo* experiments revealed a wide spectrum of effects induced by low doses of Ac-SM, which fit to the profile of non-subtype-selective $GABA_A$ agonists or positive allosteric modulators (Rudolph *et al.*, 1999; Sieghart, 2000; Olsen and Sieghart, 2009; Möhler, 2011; Sigel and Steinmann, 2012). These effects include reduction of motor activity paralleled by reduced stress-induced hyperthermia. Due to the strong motoric inhibition at higher doses (Fig. 4A) suggesting pronounced sedative effects, potential anxiolytic effects of Ac-SM (Fig. 6A-C) are difficult to assess, however, increased exploration of the open arms of the elevated-plus-maze was observed at low doses not affecting motor activity. The motor activity-independent stress-induced-hyperthermia-test

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revealed a marked dose-dependent reduction of stress response (Fig.7), supporting the assumption of anxiolytic effects induced by Ac-SM (Olivier *et al.*, 2003). Furthermore, we observed a dose-dependent increase in PTZ-induced seizure threshold (Fig.8). The long lasting reduction of activity in the home cage suggests a long biological half-life of Ac-SM (Fig.5).

Taken together, our data demonstrate that the recently identified novel $GABA_A$ receptor modulator Ac-SM enhances I_{GABA} through nine receptor subtypes with similar potency but slightly different efficacies ($\alpha_{1/2/3/5/6}\beta_{1/2/3}\gamma_{2S} \sim \alpha_1\beta_2 > \alpha_4\beta_2\gamma_{2S}$). Due to the positive $GABA_A$ allosteric modulatory effects of Ac-SM (and potentially other cycloartane glycosides, (Cicek *et al.*, 2010)), it is tempting to speculate that this $GABA_A$ receptor modulation leading to sedation and muscle relaxation contributes to the alleviation of mild to moderate symptoms in menopause reported in clinical studies with preparations from *Actaea racemosa L.*, especially when hot flushes are associated with sleep and mood disturbances (Wuttke *et al.*, 2003; Frei-Kleiner *et al.*, 2005; Osmers *et al.*, 2005; Verhoeven *et al.*, 2005; Newton *et al.*, 2006; Pockaj *et al.*, 2006; Uebelhack *et al.*, 2006; Cheema *et al.*, 2007).

Actaea racemosa L. preparations are currently standardized for their 23-epi-26-deoxyactein content (Pepping, 1999), one of the 5 main *Actaea* triterpenoids (actein, 23-epi-26-deoxyactein, cimigenol-3-*O*-β-d-xylopyranoside, cimiracemoside C and cimiracemoside F). Significant, however varying amounts of Ac-SM were recently identified in *Actaea racemosa* L. plant material (0.0137 ± 1.65% bzw. 0.0092 ± 1.97%) as well as in four *Actaea* preparations (0.004 ± 6.42% - 0.0184 ± 1.92%) that are commercially available in Austria (Cicek *et al.*, 2011). Whether these estimated amounts

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of pure Ac-SM are also sufficient to induce anxiolytic, stress-reducing, anticonvulsant and sedative effects in humans, remains, however, to be clarified. Due to the efficacy of Ac-SM and its long biological half-life (Fig.5) cumulative sedative side effects of repetitive intake of *Actaea racemosa* L. preparations might not be neglectable. In addition, *Actaea* preparations are consumed in most cases without prescription. As Ac-SM as well as other hitherto unknown components/metabolites of *Actaea racemosa* L. preparations interact amongst others with various targets in the central nervous system, potential harmful interactions with other psychoactive drugs- in particular CNS-depressant drugs- should be also carefully considered.

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Authorship contributions

Participated in research design: Khom S., Schwarzer C. and Hering S.

Conducted experiments: Strommer B., Khom S., Kastenberger I., Schwarzer C.

Contributed new reagents or analytical tools: Cicek SS., Stuppner H.

Performed data analysis: Strommer B., Khom S., Kastenberger I., Schwarzer C.

Wrote or contributed to the writing of the manuscript: Khom S., Schwarzer C., Hering S.

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Footnotes

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Figure Legends

Figure 1

Structural formula of 23-O-acetylshengmanol 3-*O*-β-d-xylopyranoside (Ac-SM)

Figure 2

Subtype-dependent modulation of $GABA_A$ receptors by Ac-SM

Concentration-response curves for the modulation of GABA-induced chloride currents (I_{GABA}) through $GABA_A$ receptors composed of **(a)** (●) $\alpha_1\beta_2\gamma_{2S}$, (■) $\alpha_1\beta_2$, **(b)** (●) $\alpha_1\beta_2\gamma_{2S}$, (□) $\alpha_2\beta_2\gamma_{2S}$, (◊) $\alpha_3\beta_2\gamma_{2S}$, (∇) $\alpha_4\beta_2\gamma_{2S}$, (○) $\alpha_5\beta_2\gamma_{2S}$, (Δ) $\alpha_6\beta_2\gamma_{2S}$, **(c)** (▼) $\alpha_1\beta_1\gamma_{2S}$, (●) $\alpha_1\beta_2\gamma_{2S}$ and (▲) $\alpha_1\beta_3\gamma_{2S}$ subunits by Ac-SM. Each data point represents the mean±SEM of at least five oocytes from two different batches. **(d)** Typical traces illustrating I_{GABA} potentiation through $\alpha_2\beta_2\gamma_{2S}$ $GABA_A$ by the indicated concentration of Ac-SM.

Figure 3

Ac-SM shifts the GABA concentration-response curve towards higher GABA sensitivity

GABA concentration-response curves for $\alpha_1\beta_3$ $GABA_A$ receptors in the absence (control,◆) and in the presence of 300μM Ac-SM (◊) are compared. Each data point represents the mean±SEM from at least three oocytes from two different oocyte batches.

Figure 4 Ac-SM dose-dependently reduces locomotor activity in the OF test

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Bars indicate in **(a)** the total distance travelled, in **(b)** the time spent in the centre, in **(c)** the number of entries to the centre and in **(d)** the distance travelled in the centre (% of the total distance) of mice treated with the indicated dose Ac-SM (mg/kg bodyweight;(grey bars) compared to vehicle-treated control littermates (white bars). Bars always represent means±SEM, number of animals per group are given in the respective bar. (*) indicates statistically significant differences with $p<0.05$, (**) $p<0.01$ to control (ANOVA with post-hoc Bonferroni analysis).

Figure 5

Ac-SM reduces locomotor-activity for 12 hours without rebound effect.

Activity (given as arbitrary activity counts) in the home cage of control (black circles) and Ac-SM-treated (20mg/kg, red circles) mice is compared. The experiment was started at 5.00 pm (=time point 0) lasting for 72h. Light-on phases of the light-dark cycle are highlighted as yellow bars on the x-axis. Each data point represents a mean±SEM from 8 mice.

Figure 6

Ac-SM displays anxiolytic effects in the EPM

Bars illustrate in **(a)** the number of open arm (OA) entries in % of the total number of entries, **(b)** the time spent in the open arms (OA) in % of the total time and in **(c)** the distance travelled in the OA in % of the total distance after application of the indicated dose in mg/kg bodyweight of Ac-SM (grey bars) in comparison to a saline-treated control group (white bars). Bars represent means±SEM, number of animals per group are given

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in the respective bar. (*) indicates statistically significant differences with $p<0.05$ to control (ANOVA with post-hoc Bonferroni analysis).

Figure 7

Ac-SM dose-dependently reduces stress-induced hyperthermia (SIH)

Bars indicate SIH (ΔT; °C) 3 hours after application of either control solution (white bars) or Ac-SM at the indicated dose (grey bars). Bars represent means±SEM, number of animals per group are given in the respective bar. (*) indicates statistically significant differences with $p<0.05$, (**) $p<0.01$ to control (ANOVA with post-hoc Bonferroni analysis).

Figure 8

Ac-SM displays anticonvulsant activity.

Bars indicate changes in seizure threshold upon pentylenetetrazole (PTZ) infusion of the indicated dose of Ac-SM (grey bars) compared to control-treated littermates (white bars). Each bar represents the mean±SEM, the number of tested animals is given in the respective bar. (**) indicates statistically significant differences with $p<0.01$, (***) $p<0.001$ to control (ANOVA with post-hoc Bonferroni analysis).

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Table 1

Summary of potencies (EC_{50}), efficiencies (maximal potentiation of I_{GABA}), Hill-coefficients (n_H) and number of experiments of Ac-SM enhancement of $GABA_A$ receptors with different subunit compositions

Subunit combination	**EC_{50} [µM]**	**max. Potentiation of I_{GABA} [%]**	**n_H**	**number of experiments**
$\alpha_1\beta_2\gamma_{2S}$	47.1 ± 20.9	1187 ± 166	1.0 ± 0.1	6
$\alpha_2\beta_2\gamma_{2S}$	39.1 ± 8.4	1454 ± 97	1.7 ± 0.3	7
$\alpha_3\beta_2\gamma_{2S}$	47.5 ± 11.7	1174 ± 218	2.0 ± 0.3	4
$\alpha_4\beta_2\gamma_{2S}$	46.3 ± 4.7	752 ± 53	1.4 ± 0.1	3
$\alpha_5\beta_2\gamma_{2S}$	50.9 ± 11.8	1408 ± 87	1.7 ± 0.4	3
$\alpha_6\beta_2\gamma_{2S}$	46.0 ± 22.9	1171 ± 274	1.0 ± 0.1	3
$\alpha_1\beta_2$	45.0 ± 11.4	1277 ± 243	1.5 ± 0.3	5
$\alpha_1\beta_1\gamma_{2S}$	43.4 ± 15.9	1069 ± 214	1.6 ± 0.4	6
$\alpha_1\beta_3\gamma_{2S}$	35.4 ± 12.3	1247 ± 222	1.7 ± 0.4	4

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Figure 1

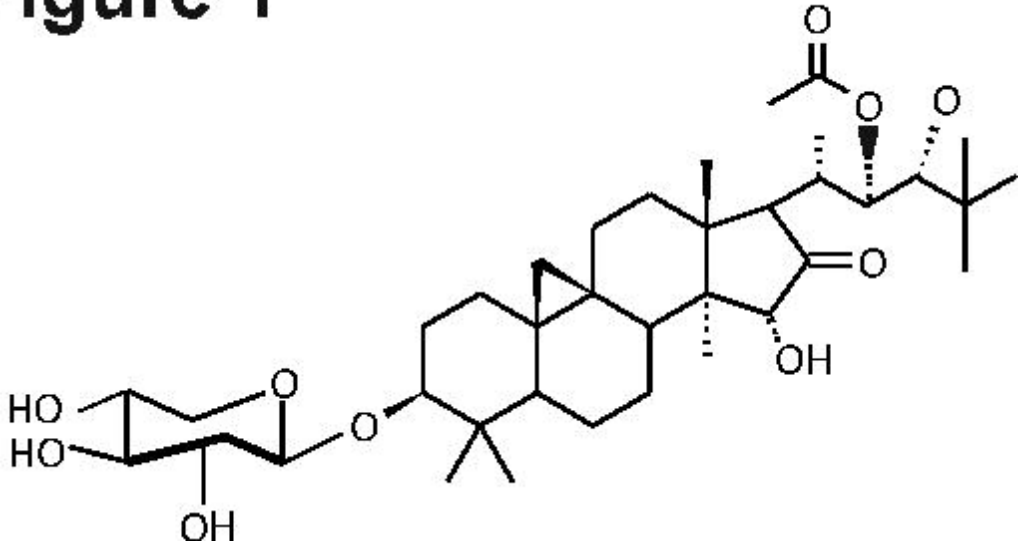


Figure 2

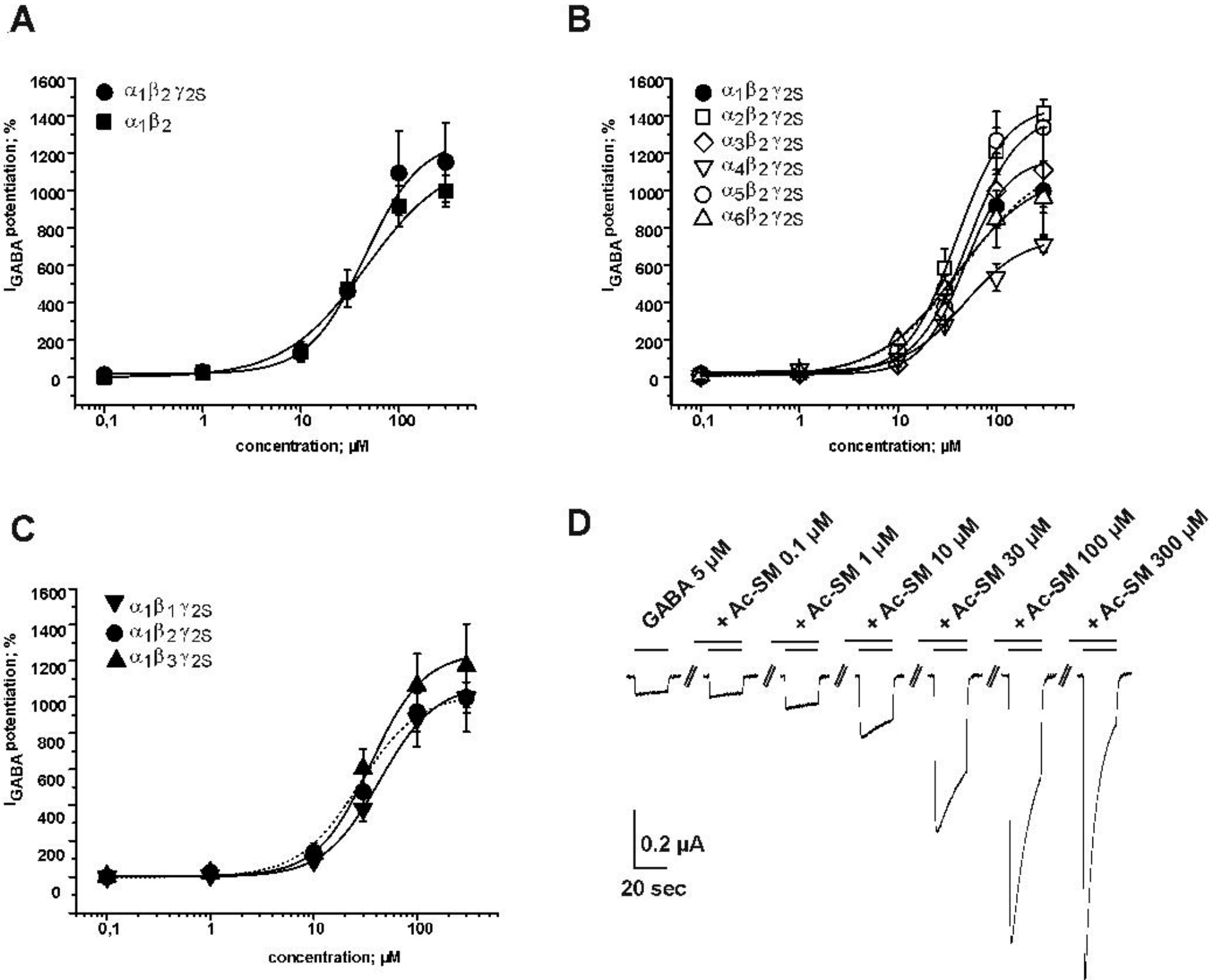

A
B
C
D
$\alpha_1\beta_2\gamma_{2S}$
$\alpha_1\beta_2$
$\alpha_2\beta_2\gamma_{2S}$
$\alpha_3\beta_2\gamma_{2S}$
$\alpha_4\beta_2\gamma_{2S}$
$\alpha_5\beta_2\gamma_{2S}$
$\alpha_6\beta_2\gamma_{2S}$
$\alpha_1\beta_1\gamma_{2S}$
$\alpha_1\beta_3\gamma_{2S}$
I_{GABA} potentiation; %
concentration; µM
0,1
1
10
100
0
200
400
600
800
1000
1200
1400
1600
GABA 5 µM
+ Ac-SM 0.1 µM
+ Ac-SM 1 µM
+ Ac-SM 10 µM
+ Ac-SM 30 µM
+ Ac-SM 100 µM
+ Ac-SM 300 µM
0.2 µA
20 sec

Figure 3

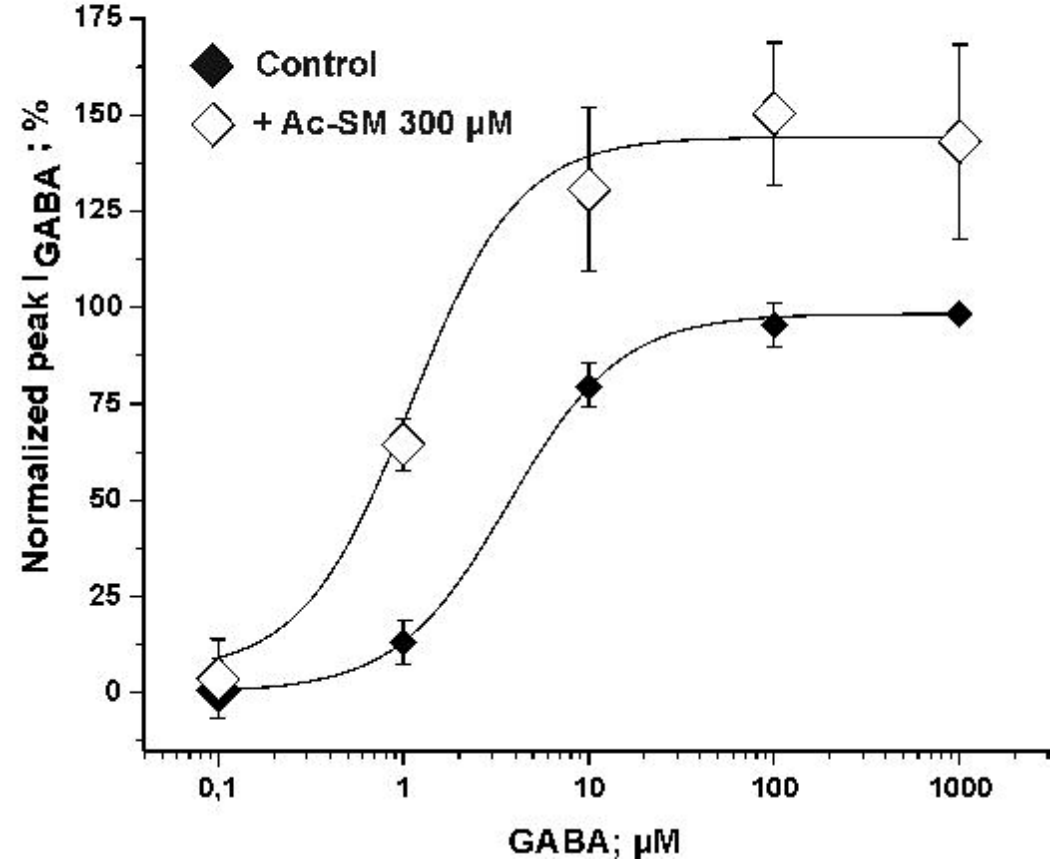

Control
+ Ac-SM 300 µM
Normalized peak I_{GABA}; %
175
150
125
100
75
50
25
0
0,1
1
10
100
1000
GABA; µM

Figure 4

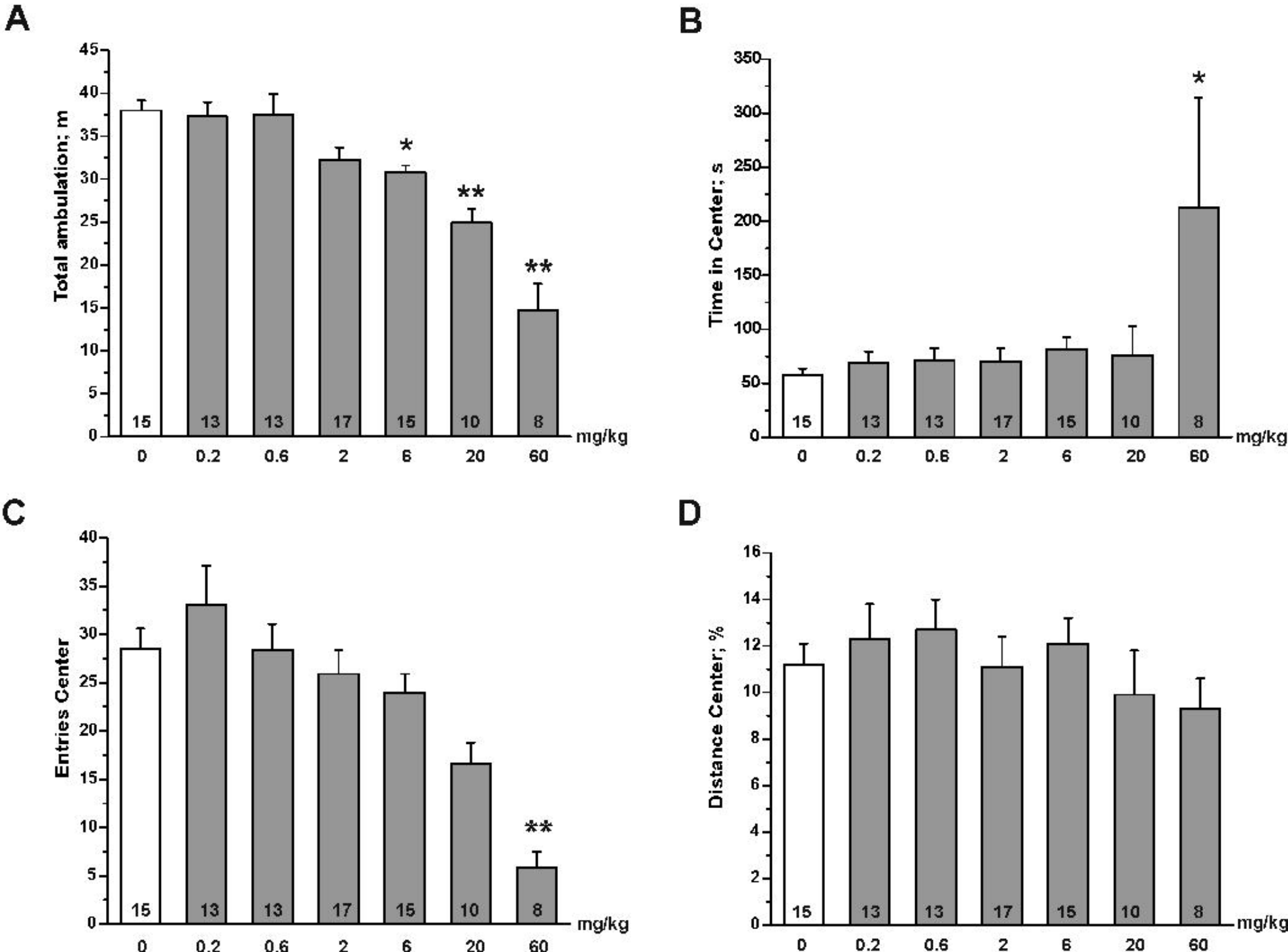

A
Total ambulation; m
45
40
35
30
25
20
15
10
5
0
15
13
13
17
15
10
8
*
**
**
mg/kg
0
0.2
0.6
2
6
20
60
B
Time in Center; s
350
300
250
200
150
100
50
0
15
13
13
17
15
10
8
*
mg/kg
0
0.2
0.6
2
6
20
60
C
Entries Center
40
35
30
25
20
15
10
5
0
15
13
13
17
15
10
8
**
mg/kg
0
0.2
0.6
2
6
20
60
D
Distance Center; %
16
14
12
10
8
6
4
2
0
15
13
13
17
15
10
8
mg/kg
0
0.2
0.6
2
6
20
60

Figure 5

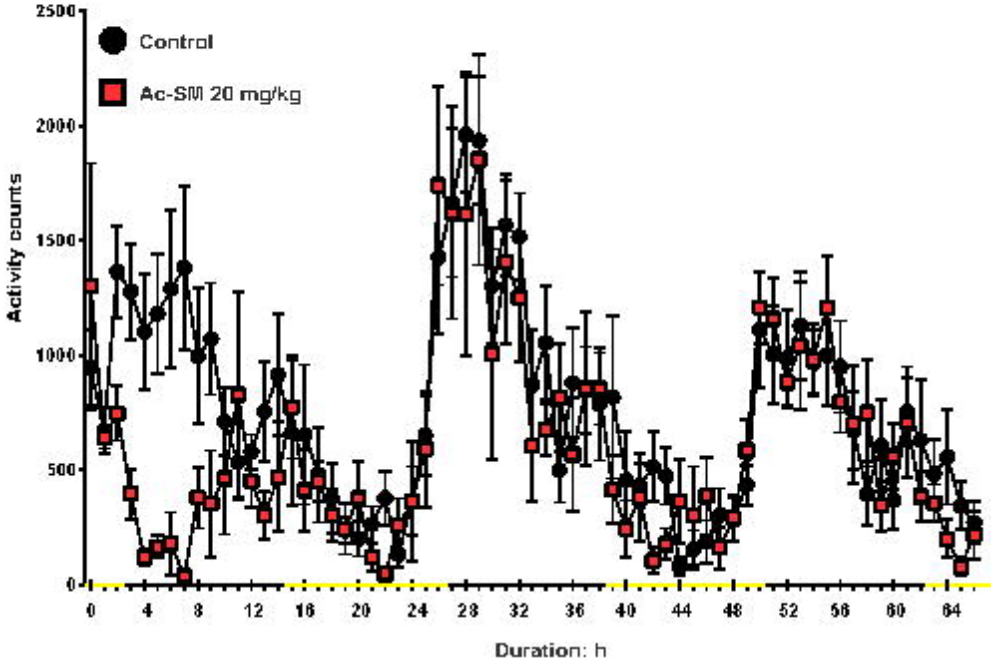

Control
Ac-SM 20 mg/kg
Activity counts
2500
2000
1500
1000
500
0
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
Duration: h

Figure 6

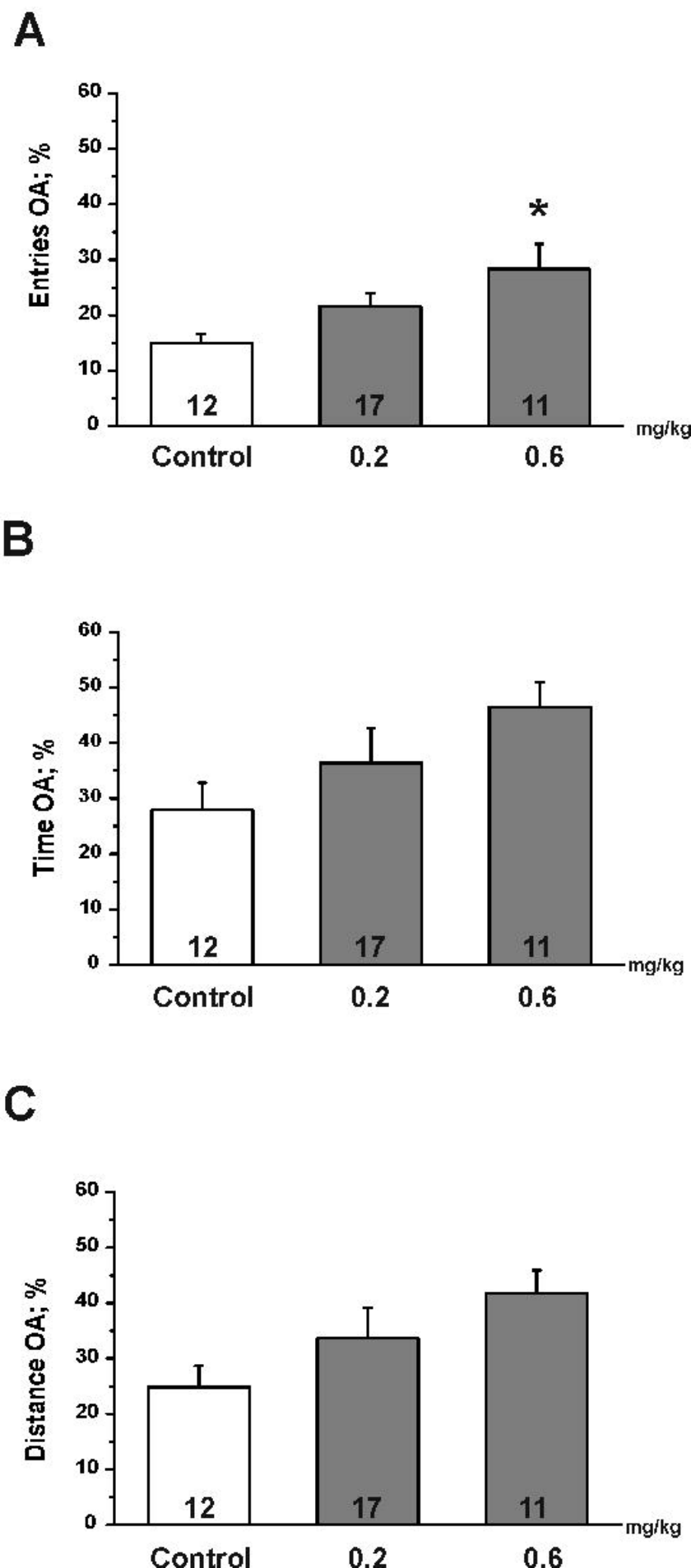

A
Entries OA; %
60
50
40
30
20
10
0
*
12
17
11
Control
0.2
0.6
mg/kg
B
Time OA; %
60
50
40
30
20
10
0
12
17
11
Control
0.2
0.6
mg/kg
C
Distance OA; %
60
50
40
30
20
10
0
12
17
11
Control
0.2
0.6
mg/kg

Figure 7

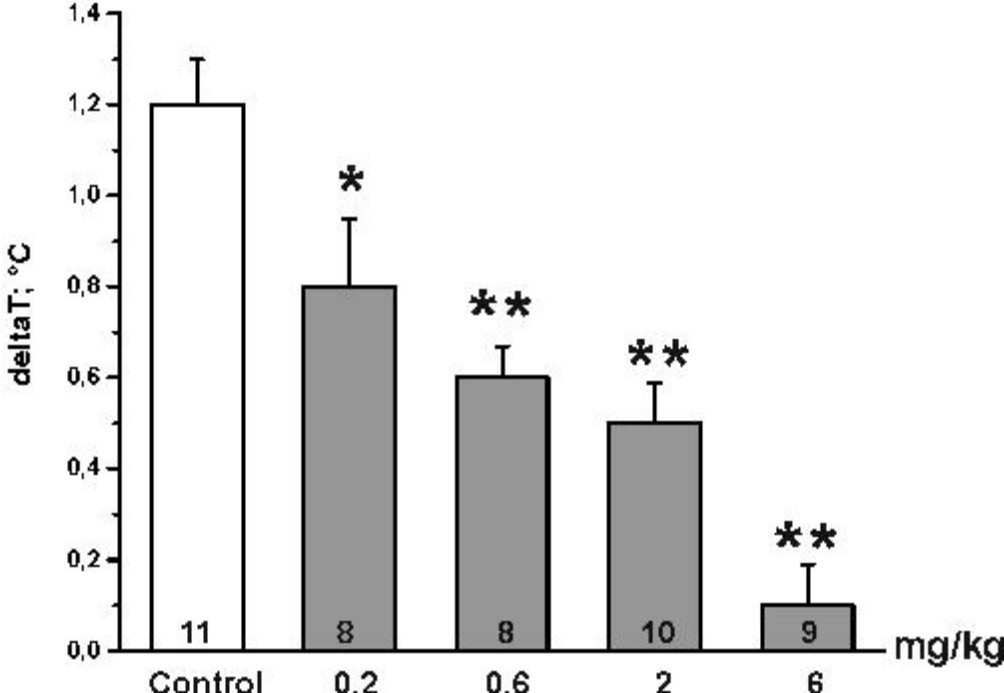
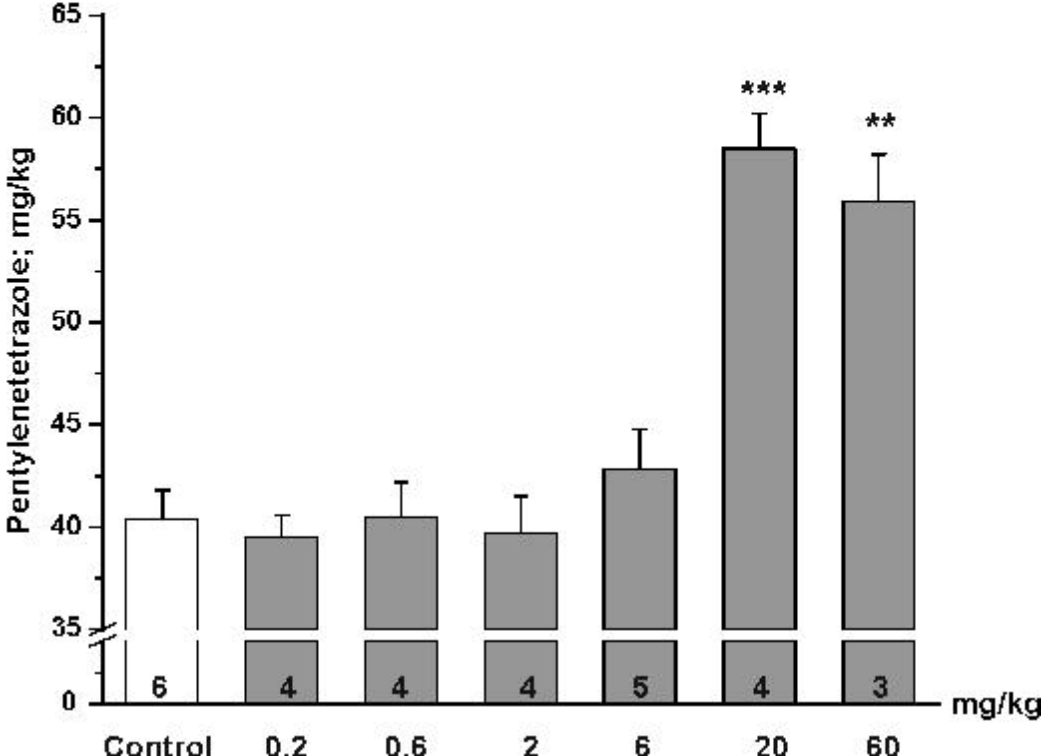


Figure 8


Pentylenetetrazole; mg/kg
65
60
55
50
45
40
35
0
***
**
6
4
4
4
5
4
3
mg/kg
Control
0.2
0.6
2
6
20
60