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# Analysis of β-subunit-dependent $GABA_A$ receptor modulation and behavioral effects of valerenic acid derivatives

Khom S., Hintersteiner J.[*], Luger D.[*] Haider M., Pototschnig G., Mihovilovic MD., Schwarzer C., Hering S.

Department of Pharmacology and Toxicology, University of Vienna, Althanstraße 14, A-1090 Vienna, Austria (S.K., J.H., D.L., S.H.)

Institute of Applied Synthetic Chemistry, TU Wien, Getreidemarkt 9, A-1060 Vienna, Austria (M.H., G.P., M.D.M.)

Department of Pharmacology, Medical University of Innsbruck, Peter-Mayr-Str. 1a, A-6020 Innsbruck, Austria (C.S.)

Running title: VA derivatives as anticonvulsants

**To whom correspondence should be addressed:**

Dr. Sophia Khom

Department of Pharmacology and Toxicology, University of Vienna,

Althanstrasse 14, A-1090 Vienna

**Tel:** 0043-1-4277-55345

**Fax:** 0043-1-4277-855345

**Email:** sophia.khom@univie.ac.at

ao. Prof. Dr. Mag. Christoph Schwarzer

Department of Pharmacology, Medical University of Innsbruck

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

**Tel:** 0043-512-9003-71205

**Fax:** 0043-512-900373200

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

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**Non-standard abbreviations**: VA (valerenic acid)

VA-A (VA-amide; (*E*)-3-((4S,7R,7aR)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl)-2-methylacrylamide),

VA-MA (VA-methylamide; (*E*)-3-((4S,7R,7aR)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl)-*N*,2-dimethylacrylamide)

VA-DMA (VA-dimethylamide; (*E*)-3-((4S,7R,7aR)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl)-*N*,*N*,2-trimethylacrylamide)

VA-EA (VA-ethylamide; (*E*)-3-((4S,7R,7aR)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl)-*N*-ethyl-2-methylacrylamide)

VA-DEA (VA-diethylamide; (*E*)-3-((4S,7R,7aR)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl)-*N*,*N*-diethyl-2-methylacrylamide)

VA-TET (VA-tetrazole; 5-((*E*)-1-((4S,7R,7aR)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl)prop-1-en-2-yl)-1*H*-tetrazole)

VA-CN (VA-nitrile; (*E*)-3-((4S,7R,7aR)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl)-2-methylacrylonitrile)

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## Abstract

Valerenic acid (VA) - a β2/3-selective γ-aminobutyric acid (GABA) type A ($GABA_A$) receptor modulator - displays anxiolytic and anticonvulsive effects in mice devoid of sedation, making VA an interesting drug candidate. Here, we analyzed β-subunit-dependent enhancement of GABA-induced chloride currents ($I_{GABA}$) by a library of VA derivatives and studied their effects on pentylenetetrazole (PTZ)-induced seizure threshold and locomotion. Compound-induced $I_{GABA}$ enhancement was determined in oocytes expressing α1β1γ2S, α1β2γ2S, or α1β3γ2S receptors. Effects on seizure threshold and locomotion were studied using C57BL/6N mice and compared to saline-treated controls. β2/3-selective VA derivatives such as VA-amide (VA-A) modulating α1β3γ2S (VA-A: $E_{max}$=972±69%, n=6, $p<0.05$) and α1β2γ2S receptors ($E_{max}$=1119±72%, n=6, $p<0.05$) more efficaciously than VA (α1β3γ2S: VA: $E_{max}$=632±88%, n=9 vs. α1β2γ2S: VA: $E_{max}$=721±68%, n=6) displayed significantly more pronounced seizure threshold elevation than VA (saline-control: 40.4±1.4mg/kg PTZ vs. VA 10mg/kg: 49.0±1.8mg/kg PTZ vs. VA-A 3mg/kg: 57.9±1.9mg/kg PTZ, $p<0.05$). Similarly, VA's methylamide (VA-MA) enhancing $I_{GABA}$ through β3-containing receptors more efficaciously than VA ($E_{max}$=1043±57%, $p<0.01$, n=6) displayed stronger anticonvulsive effects. Increased potency of $I_{GABA}$ enhancement and anticonvulsive effects at lower doses compared to VA were observed for VA-tetrazole (α1β3γ2S: VA-TET: $EC_{50}$=6.0±1.0μM, $p<0.05$; VA-TET: 0.3mg/kg: 47.3±0.5mg/kg PTZ vs. VA: 10mg/kg: 49.0±1.8mg/kg PTZ, $p<0.05$). At higher doses (≥10mg/kg), VA-A, VA-MA and VA-TET reduced locomotion. In contrast, unselective VA-derivatives induced anticonvulsive effects only at high doses (30mg/kg) or did not display any behavioral effects. Our data indicate that the β2/3-selective compounds VA-A, VA-MA and VA-TET induce anticonvulsive effects at low doses (≤10mg/kg), while impairment of locomotion was observed at doses ≥10mg/kg.

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## Introduction

Interaction of γ-aminobutyric acid (GABA) with GABA type A ($GABA_A$) receptors enables fast inhibitory neurotransmission in the mammalian brain (Barnard *et al.*, 1998; Olsen and Sieghart, 2008; Sigel and Steinmann, 2012) . Like other members of the family of pentameric ligand-gated ion channels (pLGIC), $GABA_A$ receptors are constituted by pseudosymmetrical assembly of five identical or homologous subunits forming a chloride-conducting ion (Miller and Aricescu, 2014). The human genome comprises genes encoding for 19 different $GABA_A$ receptor subunits (α1-6, β1-3, γ1-3, δ, ε, ρ1-3, π and θ (Simon *et al.*, 2004)) theoretically allowing the formation of multiple $GABA_A$ receptor subtypes. The receptor's subunit composition determines its pharmacological properties including agonist sensitivity as well as its sensitivity for drugs (Barnard *et al.*, 1998; Sigel and Steinmann, 2012). In addition, the highly specific cellular and subcellular distribution of $GABA_A$ receptor subunits/subtypes (Pirker *et al.*, 2000; Schwarzer *et al.*, 2001; Olsen and Sieghart, 2008) and in particular the assignment of therapeutic effects of commonly applied $GABA_A$ receptor modulators such as benzodiazepines (e.g. Möhler *et al.*, 2001; Rudolph *et al.*, 2001; Rudolph and Knoflach, 2011; Gallos *et al.*, 2012, 2015; Rudolph and Möhler, 2014) or anaesthetics (Jurd *et al.*, 2003) to single $GABA_A$ receptor subunits raises the possibility to develop drugs selectively targeting specific brain circuits. Such subunit-selective $GABA_A$ receptor modulators are predicted to display the desired therapeutic effects with reduced side effects.

Valerenic acid (VA) – a sesquiterpenoid compound found in common *Valerian* – selectively modulates γ-aminobutyric acid (GABA) type A ($GABA_A$) receptors containing β2- or β3-subunits, while displaying only negligible effects on $GABA_A$ receptors incorporating β1-subunits (Khom *et al.*, 2007; Benke *et al.*, 2009). *In vivo*, VA induces anxiolytic (Benke *et al.*, 2009; Khom *et al.*, 2010; Hintersteiner *et al.*, 2014) and anticonvulsive (Hintersteiner *et al.*, 2014) effects. Most notably, VA does not significantly reduce locomotor activity even at high doses (Khom *et al.*, 2010).

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These findings combined with a promising pharmacokinetic profile (Sampath *et al.*, 2012) make VA an interesting drug candidate. Previously, we have reported more pronounced anxiolytic and/or anticonvulsive effects by selected VA amide (Khom et al., 2010) and ester derivatives (Hintersteiner *et al.*, 2014). In contrast to VA ester derivatives that are significantly less active *in vitro* and thus might act as prodrugs (Hintersteiner *et al.*, 2014), both VA amide derivatives (Khom *et al.*, 2010; Kopp *et al.*, 2010) as well as VA's nitrile and tetrazole analogues (Kopp *et al.*, 2010) efficaciously enhance GABA-induced chloride currents ($I_{GABA}$) through receptors composed of α1β3 subunits (Khom *et al.*, 2010) or α1β2γ2S (Kopp *et al.*, 2010) expressed in *Xenopus* oocytes.

Therefore, to evaluate the potential of VA derivatives as scaffold for the development of novel anticonvulsants, we extended these previous studies by determining β-subunit-dependency of $I_{GABA}$ modulation by a small focused library of VA derivatives in oocytes expressing α1β1γ2S, α1β2γ2S, or α1β3γ2S receptors followed by an *in vivo* investigation of their effects on pentylenetetrazole (PTZ)-induced seizure threshold and locomotor activity in male C57BL/6N mice.

Our study shows that VA's amide (VA-A) and methylamide (VA-MA) derivatives, respectively, modulating α1β3γ2S $GABA_A$ receptors more efficaciously than VA displayed also significantly more pronounced elevation of PTZ-induced seizure threshold. In addition, VA's tetrazole derivative (VA-TET) – an efficacious VA derivative already reported in a preceding study by Kopp et al. (Kopp *et al.*, 2010) - modulating α1β3γ2S receptors more potently than VA - displayed anticonvulsive effects at lower doses than the VA. In contrast, unselective VA derivatives such VA-ethylamide (VA-EA) or VA-diethylamide (VA-DEA) displayed anticonvulsive effects only at high doses (30mg/kg) or did not show any effects on either PTZ-induced seizure threshold or locomotion (VA-dimethylamide, VA-DEA).

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## Materials and Methods

### Animals and animal welfare

All experiments involving animals were approved by the Austrian Animal Experimentation Ethics Board in compliance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes ETS no.: 123, which is in line with the EU Directive 2010/63/EU (GZ 66.006/0019-C/GT/2007, GZ 66.006/0008-II/10b/2008 and GZ 66.006/0009-II/10b/2010). Every effort was taken to minimize the number of animals used.

Female *Xenopus laevis* frogs were purchased from NASCO (Fort Atkinson, USA) and kept in groups in temperature-controlled, continuous-flow water tanks (20±1°C). Male mice (C57BL/6N) were obtained from Charles River Laboratories (Sulzfeld, Germany). 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, they were transferred to the testing facility, continuing *ad libitum* access to food and water.

The temperature in the holding (for mice and frogs) and testing facilities was fixed to 22±2°C; the humidity ranged between 40-60%; a 12h light-dark cycle was in operation (lights on from 07.00 to 19.00).

### Chemicals

All chemicals used in this study were obtained from Sigma Aldrich (Vienna, Austria) except where stated otherwise. Valerenic acid (VA) was purchased from HWI Pharma Solutions (Rülzheim, Germany). VA derivatives (structural formulae are depicted in Fig.1) were synthesized as previously described: Description of the syntheses of VA-A ((*E*)-3-((4S,7R,7aR)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl)-2-methylacrylamide), VA-MA ((*E*)-3-((4S,7R,7aR)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl)-*N*,2-dimethylacrylamide), VA-DMA ((*E*)-3-((4S,7R,7aR)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-

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1*H*-inden-4-yl)-*N*,*N*,2-trimethylacrylamide), VA-EA ((*E*)-3-((4S,7R,7aR)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl)-*N*-ethyl-2-methylacrylamide), VA-DEA ((*E*)-3-((4S,7R,7aR)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl)-*N*,*N*-diethyl-2-methylacrylamide) can be found in (Khom *et al.*, 2010) and for VA-TET (5-((*E*)-1-((4S,7R,7aR)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl)prop-1-en-2-yl)-1*H*-tetrazole), and VA-CN ((*E*)-3-((4S,7R,7aR)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl)-2-methylacrylonitrile) see Kopp *et al.*, 2010. Stock solutions (100mM for *in vitro* experiments and 1mg/10µl for *in vivo* experiments, respectively) were prepared in 100% dimethylsulfoxide (DMSO). VA and its derivatives were used up to a concentration of 500µM in *in vitro* experiments. Equal amounts of DMSO were present in control and compound-containing solutions. The maximum DMSO concentration in the bath (0.5%) did not affect $I_{GABA}$. 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.

**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 elsewhere (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). After isolation, oocytes were injected with about 10-50nl of

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nuclease-free water containing the different rat cRNAs (100-2000ng/µl/subunit). For expression of α1β3γ2S and α1β2γ2S receptors, respectively, cRNAs were mixed in a ratio of 1:1:10 (Boileau *et al.*, 2002); to avoid formation of homooligomeric β1-receptors in the case of α1β1γ2S a ratio of 3:1:10 was used (Krishek *et al.*, 1996). Electrophysiological experiments were done using the two-microelectrode-voltage-clamp-technique at a holding potential of -70mV 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, 1mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$ and 5mM HEPES (pH 7.4). Microelectrodes were filled with 2M KCl and had resistances between 1 and 3MΩ.

**Perfusion system**

GABA and drugs were applied by means of a fast perfusion system; drug or control solutions were applied by means of a TECAN Miniprep 60 permitting automation of the experiments ((Baburin *et al.*, 2006) ScreeningTool, npi electronic). To elicit $I_{GABA}$, the chamber was perfused with 120µl of GABA-containing solution at a volume rate between 300 and 1000µl/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 compound concentrations, the duration of washout periods was extended stepwise, i.e. 1min (GABA $EC_{3-7}$) to 1.5min (co-application of GABA $EC_{3-7}$ in the presence ≤1µM compound;) to 2.5min (co-application of GABA $EC_{3-7}$ in the presence of ≤10µM compound) to 5min (co-application of GABA $EC_{3-7}$ and ≤100µM compound) to 15min (GABA $EC_{3-7}$ in the presence of 300-500µM compound). Oocytes with maximal current amplitudes >5µA were discarded to exclude voltage-clamp errors.

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**Analyzing concentration-response curves**

Enhancement of chloride currents ($I_{GABA}$) by VA and VA derivatives was measured at a GABA concentration eliciting between 3 and 7% of the maximal current amplitude ($EC_{3–7}$). The $EC_{3-7}$ was determined at the beginning of the experiment for each oocyte by application of 1mM GABA followed by submaximal GABA concentrations. $I_{GABA}$ enhancement was defined as $(I_{(GABA+Comp)}/I_{GABA}) - 1$, where $I_{(GABA+Comp)}$ is the current response in the presence of compound and $I_{GABA}$ is the control GABA current. 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 following equation:

$Y = min + (max-min) * x^{nH} / (k^{nH}+x^{nH})$.

In this equation, k corresponds to the $EC_{50}$ value, x-values are logs of concentration, and $n_H$ stands for the Hill coefficient. Each data point represents the mean±SEM from ≥3 oocytes and ≥2 oocyte batches.

***In vivo* characterization of VA derivatives**

Only male C57BL/6N mice (age 3-6 months) were used in the tests described below. Intraperitoneal (i.p.) injection of control or compound-containing solutions was done 30min before the test. Indicated doses in the results and discussion section represent mg/kg bodyweight.

**Seizure threshold**

Seizure threshold was determined by pentylenetetrazole (PTZ) tail-vein infusion on freely moving animals at a rate of 100µl/min (10mg/ml PTZ in saline, pH=7.4). Infusion was stopped when animals displayed generalized clonic seizures. Animals were immediately killed by cervical displacement after onset of seizures. The seizure threshold dose was calculated from the infused dose in relation to body weight (mg/kg).

### Open-field-test

Exploration of a novel environment was tested over 10min in a 50x50cm box built from gray PVC equipped with infra-red beams. Illumination intensity was set to 150lux in the center. Animals' motor activity was analyzed using ActiMot-2 equipment and software (TSE-systems, Bad Homburg, Germany).

### Statistical analysis

Statistical significance was calculated using one-way ANOVA followed by a post-hoc mean comparison with Bonferroni (OriginLab Corporation, USA or GraphPad, La Jolla, USA). *p*-values of $<0.05$ were accepted as statistically significant. All data are given as mean±SEM.

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## Results

### Determination of β-subunit-dependency of $I_{GABA}$ modulation by valerenic acid and VA derivatives

$GABA_A$ receptors composed of α1β1γ2S, α1β2γ2S or α1β3γ2S subunits were expressed in *Xenopus laevis* oocytes and modulation of GABA-induced chloride currents ($I_{GABA}$, GABA $EC_{3-7}$) by valerenic acid (VA) and seven carboxyl-group modified derivatives was analyzed by means of the 2-microelectrode voltage clamp technique (for structural formulae of investigated VA derivatives, see Fig.1). Amidation of VA (VA-amide, VA-A) resulted in significantly stronger $I_{GABA}$ enhancement of both α1β3γ2S (VA-A: $E_{max}$=972±69%, n=6 vs. VA: $E_{max}$=632±88%, n=9 $p<0.01$) and α1β2γ2S receptors (VA-A: $E_{max}$=1119±72%, n=6 vs. VA: $E_{max}$=721±68%, n=5, $p<0.05$, compare Figs.2A and C). Subsequent mono-methylation of VA-A (VA-mono-methylamide, VA-MA) also significantly increased $I_{GABA}$ enhancement of α1β3γ2S receptors (VA-MA: $E_{max}$=1043±57%, n=5, $p<0.01$, Fig.2E) compared to VA; in addition, a strong trend towards more pronounced $I_{GABA}$ enhancement of α1β2γ2S channels by VA-MA was observed; however, this effect did not reach statistical significance (VA-MA: $E_{max}$=917±36%, n=3, $p>0.05$, Fig. 2E). Modulation of $I_{GABA}$ through β1-containing receptors by VA-A and VA-MA did not significantly differ – even though slightly enhanced – from $I_{GABA}$ enhancement by VA (α1β1γ2S: VA: $E_{max}$=111±16%, n=8 vs. VA-A: $E_{max}$=218±78%, n=6; VA-MA: $E_{max}$=387±56%, n=5; $p>0.05$; compare Figs.2A, C and E; see also Table 1, see also (Khom *et al.*, 2010; Kopp *et al.*, 2010) for VA-A enhancement of α1β1-3, α1β2γ2S, and α3β3γ2S and VA-MA enhancement of α1β3 and α1β2γ2S receptors).

Replacement of the carboxyl-moiety by the bioisosteric tetrazole group (VA-TET) significantly increased efficacy on α1β2γ2S ($E_{max}$=1091±87%, n=5, $p<0.05$, Fig.2G) receptors though with a slightly reduced potency compared to VA, while efficacy of $I_{GABA}$ enhancement on α1β1γ2S ($E_{max}$=176±43%, n=7) and α1β3γ2S ($E_{max}$=668±57%, n=8; Fig.2G) receptors did not differ from that of the parent compound. However, most remarkably, VA-TET displayed

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an approximately 3-fold increased potency on β3-containing receptors compared to VA (VA: $EC_{50}$=20.2±5.2μM; n=9 vs. VA-TET: $EC_{50}$=6.0±1.0μM; n=8; see Fig.2 for representative current traces for β-subunit-dependent $I_{GABA}$ enhancement by (B) VA, (D) VA-A, (F) VA-MA and (H) VA-TET; data for $I_{GABA}$ enhancement of $GABA_A$ channels composed of α1β3γ2S subunits by VA are taken from (Luger *et al.*, 2015); see also the effect of VA-TET applied at low GABA concentrations ($EC_{1-4}$) in (Kopp *et al.*, 2010).

Similarly, substituting VA's carboxyl group by a nitrile (VA nitrile, VA-CN) did not affect β2/3-subunit-selective $I_{GABA}$ potentiation: $I_{GABA}$ enhancement by VA-CN through α1β1γ2S ($E_{max}$=55±14%, n=7), α1β2γ2S ($E_{max}$=765±117%, n=4), and α1β3γ2S ($E_{max}$=522±114%, n=7) channels, respectively, was similar to that of VA; however, a trend towards slightly decreased potency on β2- and β3-subunit containing receptors was observed (see Fig.3A; see also (Kopp *et al.*, 2010) for $I_{GABA}$ enhancement through α1β2γ2S channels by VA-CN at GABA $EC_{1-4}$ concentrations).

In contrast, introducing bulkier residues such as mono-ethylamide (VA-EA), di-methylamide (VA-DMA) or di-ethylamide (VA-DEA) significantly elevated efficacy on $\beta_1$-containing receptors compared to VA (VA-EA: $E_{max}$=458±124%, n=5, Fig.3B; VA-DMA: $E_{max}$=305±67%, n=6, Fig.3C; VA-DEA: $E_{max}$=318±84%, n=7, Fig.3D; $p>0.05$), while the estimated efficacies on β2- and β3-containing receptors were comparable to VA, indicating a loss of subunit-selectivity (Figs.3B-D). Data for maximal $I_{GABA}$ enhancement ($E_{max}$, %), $EC_{50}$ (μM), Hill-coefficients ($n_H$) and number of experiments (n) for all receptor subunit compositions tested are summarized in Table 1.

**Effects of VA and VA derivatives on pentylenetetrazole (PTZ)-induced seizure threshold**

We have recently reported that the elevation of PTZ-induced seizure threshold by VA (3mg/kg bodyweight) is most pronounced 30min after application (Hintersteiner *et al.*, 2014). In the present study, dose-dependent effects of VA on seizure threshold were determined. As

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depicted in Fig.4A, VA did not alter seizure threshold at doses <3mg/kg bodyweight; in contrast, pronounced seizure threshold elevation was observed after application of VA at a dose of 3 or 10mg/kg bodyweight (control: 40.4±1.4 mg/kg PTZ, n=6 vs. VA 3mg/kg: 47.7±1.4mg/kg PTZ, n=4, $p<0.01$ and VA 10mg/kg: 49.0±1.8mg/kg PTZ, n=4; $p<0.05$, respectively; data for seizure threshold elevation by VA at a dose of 3mg/kg bodyweight are taken from (Hintersteiner *et al.*, 2014)). Seizure threshold of animals treated with VA at a dose of 30mg/kg bodyweight did not differ significantly from diluent-treated control animals (30mg/kg VA: 43.4±1.8mg/kg PTZ, n=3, $p>0.05$; Fig.4A). Compared to VA, VA-A exerted significantly stronger anticonvulsive activity at doses ≥3mg/kg (see Fig.4B, VA-A 3mg/kg: 57.9±1.9mg/kg PTZ, n=4; VA-A 10mg/kg: 55.4±0.7mg/kg PTZ, n=4, $p<0.001$). Like VA, VA-A also displayed a trend towards reduced activity at higher doses (30mg/kg), however – in contrast to VA – still retained its anticonvulsive potential (Fig.4B, VA-A 30mg/kg: 50.6±2.2mg/kg PTZ, n=3, $p<0.01$).

A similar (but left-shifted) bell-shaped dose-response curve on PTZ-induced seizure threshold was observed for VA-TET: at a dose of 0.3mg/kg bodyweight (Fig.4C: VA-TET 0.3mg/kg: 47.3±0.5mg/kg PTZ, n=5, $p<0.05$) VA-TET's anticonvulsive activity was comparable to that of VA at 10-fold higher doses (3mg/kg) indicating a significantly increased potency. However, VA-TET lost its anticonvulsive properties at doses ≥1mg/kg. The methylated VA-A derivative (VA-MA) induced the most pronounced increase in seizure threshold of all tested compounds; first significant effects were observed at a dose of 10mg/kg (Fig.4D: VA-MA 10mg/kg: 50.4±1.4mg/kg PTZ, n=4, $p<0.001$). In contrast to VA, VA-A and VA-TET, application of higher doses (i.e. 30mg/kg) VA-MA resulted in an even further elevated seizure threshold (VA-MA 30mg/kg: 63.6±2.5mg/kg PTZ, n=3, $p<0.001$).

As shown in Fig.4F, G and H, higher doses of VA-EA, VA-DEA, and VA-CN were required for seizure threshold elevation similar to that of VA (compare VA 3mg/kg: 47.7±1.4mg/kg PTZ, n=4, Fig.4A vs. VA-EA 30mg/kg: 55.6±0.4 mg/kg PTZ, n=4, Fig.4F vs. VA-DEA:

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30mg/kg: 48.7±1.7mg/kg PTZ, n=3, Fig.4G vs. VA-CN 10mg/kg: 51.1±0.6mg/kg PTZ, n=3, Fig.4H).

Seizure threshold of mice treated with VA-DMA (Fig.4E) did not significantly differ at any tested dose from diluent-treated control littermates.

**Effects of VA and derivatives on locomotion in the open field test**

As illustrated in Fig.5A, locomotor activity of VA-treated mice did not differ significantly from control animals at any tested dose in the open field test (control: 38.3±1.5m, n=25 vs. VA 1mg/kg: 34.9±1.3m, n=12 vs. VA 3mg/kg: 38.7±2.1m, n=16 vs. VA 10mg/kg: 38.0±1.4 m, n=16 vs. VA 30mg/kg: 37.6±2.5m, n=16).

Application of VA derivative VA-A at doses of 1mg/kg and 3mg/kg, respectively, did also not affect the total distance covered compared to control littermates; however, reduced locomotor activity in the open field test was measured after application of VA-A at doses ≥10mg/kg (control: 38.3±1.5m, n=25 vs. VA-A 10mg/kg: 29.9±2.6m, n=11, $p<0.05$ vs. VA-A 30mg/kg: 23.5±2.2m, n=18, $p<0.001$; see Fig.5B).

Like VA-A, derivatives VA-TET (Fig.5C), VA-MA (Fig.5D) and VA-CN (Fig.5E) did not affect locomotor activity at low doses (≤10mg/kg), however at higher doses reduced ambulation was observed for these compounds (control: 38.3±1.5m, n=25 vs. VA-TET 30mg/kg: 19.2±2.1m, n=13, $p<0.001$ vs. VA-MA 30mg/kg: 29.7±2.0m, n=12, $p<0.01$ vs. VA-CN 30mg/kg: 31.5±1.5m, n=14, $p<0.05$).

Finally, analysis of total distance did not reveal any significantly different behavior of animals treated with any dose of the β-subunit-unselective VA derivatives VA-DMA (Fig.5F), VA-EA (Fig.5G) and VA-DEA (Fig.5H) or diluent-treated control animals.

## Discussion

Subunit-selective $GABA_A$ receptor modulators represent interesting lead structures for drug development. The natural compound valerenic acid (VA) selectively modulates $GABA_A$ receptors containing either β2- or β3-subunits with only residual modulatory activity on $GABA_A$ receptors incorporating β1-subunits at high concentrations (Khom *et al.*, 2007; Benke *et al.*, 2009). As opposed to benzodiazepines, VA and also its derivatives do not interact with the benzodiazepine binding site (i.e. $I_{GABA}$ enhancement by VA and VA derivatives does not require the presence of a γ2S-subunit and VA's modulatory action cannot be blocked by the benzodiazepine site antagonist flumazenil (Khom *et al.*, 2007, Khom *et al.*, 2010)). This selectivity profile combined with pronounced anticonvulsive effects (Hintersteiner *et al.*, 2014) accompanied by anxiolytic activity (Khom *et al.*, 2010; Hintersteiner *et al.*, 2014) and devoid of significant impairment of locomotor activity suggesting lack of sedative side effects makes VA and potentially also its derivatives promising drug candidates.

Previously, we and others have reported more efficacious and/or potent modulation of $GABA_A$ receptors expressed in *Xenopus* oocytes by carboxyl-modified VA derivatives (Khom *et al.*, 2010; Kopp *et al.*, 2010). Furthermore, more pronounced anxiolytic effects by the amide derivative of VA (VA-A) (Khom *et al.*, 2010) as well as stronger anxiolytic and anticonvulsive effects of VA ester derivatives compared to VA were observed (Hintersteiner *et al.*, 2014).

To further evaluate the potential of VA and carboxyl-modified derivatives (for structural formulae, see Fig.1) as scaffolds for the development of novel anticonvulsants with limited impairment of locomotor activity, we extended previous *in vitro* studies of these compounds (Khom *et al.*, 2010; Kopp *et al.*, 2010) by determining their β-subunit-dependency of $I_{GABA}$ modulation (α1β1γ2S, α1β2γ2S, α1β3γ2S) followed by a subsequent analysis of their effects on PTZ-induced seizure threshold and locomotion.

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***VA derivatization has dual effects on β-subunit-selectivity***

An interesting finding of the present study was that VA derivatization had - depending on the substituent introduced - dual effects on β-subunit-selectivity: Amidation of VA (VA-A) as well as mono-methylation of the amide (VA-MA), respectively, strongly increased efficacy of $I_{GABA}$ enhancement of β2- and β3-containing receptors compared to VA (compare Fig.2A, 2C and 2E), while efficacy of $I_{GABA}$ enhancement through β1-containing receptors did not differ significantly from that of VA, thus boosting β-subunit-selectivity. Replacing the carboxyl-function by a tetrazole group (VA-TET) significantly increased potency of $I_{GABA}$ enhancement through β3-containing receptors and efficacy of $I_{GABA}$ of β2-containing receptors, while modulation of β1-receptors by VA-TET was comparable to that of VA (compare Figs.2A and 2C). Intriguingly, this structural modification (replacement of the carboxyl-group by a tetrazole moiety) altered current kinetics (see Fig. 2H for typical $I_{GABA}$ in the presence of 10μM VA-TET): compared to $I_{GABA}$ modulated by any of the other studied VA derivatives (Figs. 2 and 3, right panels), receptor activation in the presence of VA-TET appeared to be slowed (see longer time required to reach peak currents) and in addition, current desensitization could not be observed even at high drug concentrations. The parent compound VA – at concentrations ≥30μM – has been reported to directly activate $GABA_A$ channels in the absence of GABA (see (Khom *et al.*, 2007). Compared to $I_{GABA}$, both activation and deactivation rates of these VA-induced currents were remarkably slower. We speculate that VA-TET – representing the more potent bioisoster of VA in terms of modulatory activity (see Table 1 and Fig. 2A and G)- might also directly activate the channel like VA at lower concentrations and that the observed kinetics reflect a combination of direct activation and modulatory activity. However, these differences in current kinetics warrant further investigations.

Substituting the carboxyl-group by a nitrile function (VA-CN) resulted in a similar β2/3-subuinit dependency as observed for VA (Fig.3A). In contrast, introduction of bulkier

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residues such as dimethylamide (VA-DMA), ethylamide (VA-EA) or diethylamide (VA-DEA) did not alter efficacy or potency of $I_{GABA}$ enhancement of β2- or β3-containing receptors compared to VA, but these compounds efficaciously modulated β1-containing receptors indicating a loss of β-subunit-selectivity (see Figs.3B-D).

***Evaluation of anticonvulsive effects and potential impairment of locomotor activity by VA derivatives***

In line with the stronger $I_{GABA}$ enhancement by VA-A *in vitro*, VA-A also induced a more pronounced seizure threshold elevation compared to VA (compare Fig.4A, 4B and 4D). However, in contrast to VA that did not affect locomotor activity even at doses up to 30mg/kg compared to saline-treated control animals, application of VA-A (≥10mg/kg) significantly reduced locomotor activity. This finding suggests that VA-A, as opposed to VA, also induces sedative effects (compare Figs. 5A and 5B). A similar profile was observed for VA-MA: compared to VA, $I_{GABA}$ enhancement of β3- and β2-containing receptors *in vitro* was more pronounced, and stronger anticonvulsive effects were also accompanied by a reduction of locomotor activity at higher doses (30mg/kg).VA-TET displaying *in vitro* significantly more potent $I_{GABA}$ enhancement of β3-containing receptors (efficacy comparable to VA) and more efficacious $I_{GABA}$ enhancement of β2-receptors, elevated PTZ-induced seizure threshold at 10-fold lower doses than VA (VA-TET: 0.3mg/kg: 47.3±0.5mg/kg PTZ vs. VA 3mg/kg: 47.7±1.4mg/kg PTZ (Fig.4C), but also induced the strongest reduction of locomotor activity of all tested VA derivatives at a dose of 30mg/kg (Fig.5C). These presumably sedative effects by VA-TET were, however, observed at doses more than 100-fold higher than those required for anticonvulsive effects (0.3mg/kg; see also Figs. 4C and 5C).

Efficacy and potency of $I_{GABA}$ enhancement (α1β1-3γ2S) by the nitrile derivative (VA-CN) did not significantly differ from that of VA. A trend towards – although not reaching statistical significance – reduced potency for β1- and β3-containing receptors compared to VA was observed (Fig.3A). However, higher doses of VA-CN (10mg/kg) were required for

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seizure threshold elevation (Fig.4H) and as opposed to VA, VA-CN also slightly reduced locomotion at high doses (30 mg/kg; Fig.5E).

Unaffected locomotor activity and either no effect on PTZ-induced seizure threshold or anticonvulsive effects occurring at rather high doses (30mg/kg) were observed for the unselective VA derivatives (i.e. comparable efficacy and potency on α1β1-3γ2S receptors) VA-EA (Fig.4F and 5G) and VA-DEA (Fig.4G and 5H) and VA-DMA (Figs.4E and 5F).

These findings – β2/3-selective VA derivatives displaying anticonvulsive effects at low doses (≤10mg/kg) and sedative effects at higher doses (≥10mg/kg) while unselective VA derivatives (VA-EA, VA-DEA) are anticonvulsive at higher doses (30mg/kg) without affecting locomotor activity - prompted us to probe a possible link between β-subunit selectivity and behavioral effects of this set of VA derivatives. Indeed, plotting maximal seizure threshold elevation (maximal effect or the effect reached at a dose of 30 mg/kg) versus the efficacy of $I_{GABA}$ enhancement at different subunit compositions *in vitro* revealed a significant correlation between the compounds' efficacy at β3-containing receptors and anticonvulsive effects (for details, see Supplementary data). Derivatives enhancing $I_{GABA}$ at β3-containing receptors more efficaciously than VA also displayed stronger protection against PTZ-induced seizures ($r$=0.8785; $p<0.01$, see Fig. 1S Supplementary data). Conversely, efficacy at neither β2- nor β1-containing receptors correlated with the extent of seizure threshold elevation. However, VA derivatives modulating β2-containing receptors more efficaciously also induced more pronounced impairment of locomotor activity ($r$=-0.9262; $p<0.001$, Fig. 1S Supplementary data), while neither efficacy nor potency of VA and its derivatives on β1- or β3-containing receptors apparently correlated with occurring sedation. Therefore, it is tempting to speculate that the motor-impairing effects (probably sedation) of VA derivatives are determined by the interaction with β2-containing receptors, while their anticonvulsive effects might be mediated by receptors containing β3-subunits. This hypothesis would also be in line with previous studies highlighting the role of β2-containing receptors in mediating sedative effects of

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$GABA_A$ receptor modulators such as loreclezole (Groves *et al.*, 2006), etomidate (Reynolds *et al.*, 2003; Zeller *et al.*, 2005) or even benzodiazepines (Antkowiak, 2015). In contrast, anticonvulsive effects of $GABA_A$ receptor ligands apparently might result from a broader, less selective $GABA_A$ receptor modulation including more than a single $GABA_A$ receptor subtype: Rudolph et al. have shown that anticonvulsive effects of diazepam are only partially blunted in α1H101R mice indicating that also α2, α3 and/or α5-containing $GABA_A$ receptors could contribute to anticonvulsive effects *in vivo* (Rudolph *et al.*, 1999; Löw *et al.*, 2000; Crestani *et al.*, 2002; Fradley *et al.*, 2007). In addition, the β2/3-selective $GABA_A$ receptor ligand loreclezole retained – even though reduced- seizure-protecting activity in β2N265S mice suggesting a complementary role for β3-containing receptors in mediating anticonvulsive effects (Groves *et al.*, 2006). Most notably, the expression of β3-subunits is particularly high in dendritic regions of the hippocampus and dentate gyrus (Sperk *et al.*, 1997; Miralles *et al.*, 1999; Hörtnagl *et al.*, 2013). Assuming the fundamental role of the hippocampus in seizure activity and epilepsy (Schwartzkroin, 1994; Coulter *et al.*, 2011), amplifying GABAergic neurotransmission in the hippocampus via β3-containing receptors might represent an appealing approach to develop novel anticonvulsants with reduced side effects including sedation.

However, it cannot be ruled out that the observed differences in effects on PTZ-induced seizure threshold and locomotion might be also due to distinct pharmacokinetic properties (e.g. tissue distribution, penetration of the blood-brain barrier, different onset of *in vivo* effects etc.). To answer this question warrants further studies. Furthermore, interaction of VA and its derivatives with targets for anticonvulsive acting drugs other than $GABA_A$ receptors has not been analyzed in the present study. VA has previously been reported to bind to 5-$HT_{5A}$-receptors (Dietz *et al.*, 2005) as well as metabotropic group I/II glutamate receptors (Del Valle-Mojica *et al.*, 2011), and to attenuate physical and psychological stress responses by decreasing the turnover of serotonin to 5-hydroxyindoleacetic acid and turnover of

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norepinephrine to 3-methoxy-4-hydroxyphenylethyleneglycol sulfate in rodent hippocampus and amygdala (Jung *et al.*, 2015). Thus, both anticonvulsive and sedative effects of the studied compounds might also result from interactions of VA and its derivatives with multiple drug targets. Benke et al. (2009) demonstrated ,however, that VA's anxiolytic effect in mice is mediated exclusively by interaction with β3-subunit-containing $GABA_A$ receptors based on their observation that VA-induced reduction of anxiety-related behavior was completely absent in β3N265M mice- a point mutation well-known to abolish $I_{GABA}$ enhancement by VA and other β2/3-selective $GABA_A$ receptor modulators (Groves *et al.*, 2006; Benke *et al.*, 2009). In line with this, the efficacy of the derivates on $GABA_A$ receptors *in vitro* highly correlates with the strength of anticonvulsive effects *in vivo* (this study), supports the hypothesis that these action are likely to be mediated via $GABA_A$ receptors.

Taken together, our study demonstrates that modifications of VA's carboxyl function can profoundly alter potency, efficacy and β-subunit-selectivity of VA derivatives: Replacing the carboxyl group by a tetrazole (VA-TET) (see also Kopp et al. (Kopp *et al.*, 2010)) as well as amidation (unsubstituted or mono-methylated amide (Khom *et al.*, 2010; Kopp *et al.*, 2010)) significantly increased efficacy and/or potency of $I_{GABA}$ enhancement of β2/3-containing receptors, and – most importantly – enhanced β2/3-selectivity. In contrast, introduction of bulkier substituents (as ethyl for VA-EA, di-methyl for VA-DMA, or diethyl for VA-DEA) resulted in significantly increased efficacy on β1-containing channels and thus abolished β-subunit selectivity. Increased efficacy and/or potency of $I_{GABA}$ enhancement of β2/3-containing receptors by VA derivatives VA-A, VA-MA and VA-TET is likely to result in more pronounced anticonvulsive effects at low doses (≤10mg/kg), while sedative effects occur at higher doses (≥10mg/kg). In contrast, relatively high doses (30mg/kg) of unselective VA derivatives were required for anticonvulsive effects. Our data thus suggest that VA and β2/3-selective VA derivatives represent interesting scaffolds for the development of novel anticonvulsants.

## Authorship contributions

***Participated in research design:***

Khom S., Schwarzer C. and Hering S.

***Conducted experiments:***

Khom S., Hintersteiner J., Luger D., Schwarzer C.

***Contributed new reagents or analytical tools:***

Haider M., Pototschnig G., Mihovilovic MD.

***Performed data analysis:***

Khom S., Luger D., Schwarzer C.

***Wrote or contributed to the writing of the manuscript:***

Khom S., Luger D., Schwarzer C., Mihovilovic MD., Hering S.

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## Footnotes

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* These authors contributed equally (J.H. and D.L.).

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# Legends for figures

## Figure 1

***VA and VA derivatives***

Structural formulae of studied valerenic acid (VA) and VA derivatives are illustrated.

## Figure 2

***β-subunit-dependent $I_{GABA}$ enhancement by VA and VA derivatives***

Concentration-dependent modulation of $GABA_A$ receptors composed of α1β3γ2S (dashed line), (■) α1β2γ2S and (♦) α1β1γ2S subunits by **(A)** VA (data for enhancement of α1β3γ2S receptors taken from (Luger *et al.*, 2015) **(C)** VA-A, **(E)** VA-MA, and **(G)** VA-TET.

Data were fitted by non-linear regression, as described in Materials and Methods ($I_{GABA}$ enhancement by VA-A at 300µM (red symbol) was excluded from the fit). Maximal potentiation of $I_{GABA}$ ($E_{max}$), $EC_{50}$ values, Hill-coefficients ($n_H$) and number of experiments for each compound on α1β1γ2S, α1β2γ2S and α1β3γ2S receptors are summarized in Table 1. Each data point represents a mean±S.E.M from at least 3 different oocytes from 2 different frog batches. $I_{GABA}$ potentiation at 300µM (α1β3γ2S receptors, grey symbols) for VA and VA-A, respectively, were excluded from the fit. Typical traces for the enhancement of GABA-induced chloride currents ($I_{GABA}$, $EC_{3-7}$, single bar) by 10µM of **(B)** VA, **(D)** VA-A, **(F)** VA-MA and **(H)** VA-TET (double bar; indicating co-application of GABA and compound) at the indicated $GABA_A$ receptor subtype are illustrated.

## Figure 3

***β-subunit-dependent $I_{GABA}$ modulation by VA derivatives***

Concentration-response curves for the $I_{GABA}$ enhancement through (●) α1β3γ2S, (■) α1β2γ2S and (♦) α1β1γ2S channels by VA derivatives **(A)** VA-CN, **(B)** VA-EA, **(C)** VA-DMA and

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**(D)** VA-DEA. Data were fitted by non-linear regression, as described in Materials and Methods. Maximal potentiation of $I_{GABA}$ ($E_{max}$), $EC_{50}$ values, Hill-coefficients ($n_H$) and number of experiments for each compound on α1β1γ2S, α1β2γ2S and α1β3γ2S receptors are summarized in Table 1. Each data point represents a mean±S.E.M from at least 3 different oocytes from 2 different frog batches. Typical current traces for the enhancement of GABA-induced chloride currents ($I_{GABA}$, $EC_{3-7}$, single bar) by 10μM of **(B)** VA-CN, **(D)** VA-EA, **(F)** VA-DMA and **(H)** VA-DEA (double bar; indicating co-application of GABA and compound) at the indicated $GABA_A$ receptor subtype are illustrated.

**Figure 4**

***Anticonvulsive effects of VA and VA derivatives***

Elevation of seizure threshold upon tail-vain infusion of PTZ 30 min after i.p. application of **(A)** VA is illustrated. The dotted line represents the averaged seizure threshold of saline-treated control animals. Effect on PTZ-induced seizure threshold 30 min. after i.p. application by the VA derivatives **(B)** VA-A, **(C)** VA-TET, **(D)** VA-MA, **(E)** VA-DMA, **(F)** VA-EA, **(G)** VA-DEA and **(H)** VA-CN is compared to VA (dashed line, white circles) and control animals (dotted line). Each data point represents a mean ± S.E.M from at least 3 mice. Statistical significance (p-values <0.05 were accepted as significant; *=p<0.05, **=p<0.01 and ***=p<0.001) against VA was calculated by one-way ANOVA followed by a Bonferroni post-hoc mean comparison.

**Figure 5**

***Effects on locomotion by VA and VA derivatives in the open field test (OF) test***

Black bars indicate the total distance covered in the OF test 30 min after i.p. application of **(A)** VA **(B)** VA-A, **(C)** VA-TET, **(D)** VA-MA, **(E)** VA-CN, **(F)** VA-DMA, **(G)** VA-EA and **(H)** (VA-DEA) at the indicated doses compared to saline-treated control animals (white bars

in all panels). Each bar represents a mean ± S.E.M from at least 10 different mice. Statistical significance (p-values <0.05 were accepted as significant; *=p<0.05 and ***=p<0.001) against saline-treated control animals was calculated by one-way ANOVA followed by a Bonferroni post-hoc mean comparison.

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**Table 1**

Summary of pharmacological parameters of $I_{GABA}$ enhancement through $\alpha_1\beta_1\gamma_{2S}$, $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_1\beta_3\gamma_{2S}$ receptors including maximal $I_{GABA}$ enhancement ($E_{max}$), half-maximal effective concentrations ($EC_{50}$), Hill-coefficients ($n_H$) and number of experiments for each compound at the tested subunit combinations. Data for $I_{GABA}$ enhancement of α1β3γ2S receptors by VA at GABA $EC_{3\text{-}7}$ concentrations taken from (Luger *et al.*, 2015).

**α1β1γ2S**

| | $E_{max}$ (%) | $EC_{50}$ (µM) | $n_H$ | n |
|---|---|---|---|---|
| **VA** | 111±16 | 74.4±19.3 | 1.6±0.5 | 8 |
| **VA-A** | 218±78 | 66.6±34.6 | 1.8±0.6 | 6 |
| **VA-MA** | 387±56 | 58.1±16.5 | 1.6±0.3 | 5 |
| **VA-DMA** | 305±67 | 52.5±20.1 | 1.7±0.5 | 6 |
| **VA-EA** | 458±124 | 51.4±19.8 | 1.6±0.3 | 5 |
| **VA-DEA** | 318±84 | 97.2±33.8 | 1.8±0.3 | 7 |
| **VA-CN** | 55±14 | 73.0±38.7 | 1.7±0.7 | 7 |
| **VA-TET** | 176±43 | 23.5±10.9 | 1.8±0.6 | 7 |

**α1β2γ2S**

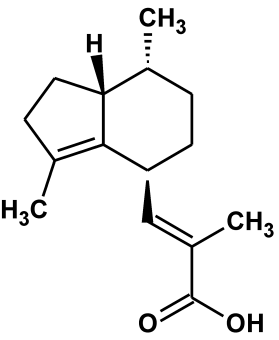
| | $E_{max}$ (%) | $EC_{50}$ (µM) | $n_H$ | n |
|---|---|---|---|---|
| **VA** | 721±68 | 23.1±4.2 | 1.4±0.2 | 5 |
| **VA-A** | 1119±72 | 14.0±2.2 | 1.4±0.2 | 6 |
| **VA-MA** | 917±36 | 9.1±1.4 | 1.5±0.1 | 3 |
| **VA-DMA** | 594±36 | 63.9±23.7 | 1.3±0.2 | 5 |
| **VA-EA** | 554±49 | 11.0±3.6 | 1.6±0.3 | 3 |
| **VA-DEA** | 573±33 | 54.4±11.1 | 1.6±0.3 | 5 |

| | | | | |
|---|---|---|---|---|
| **VA-CN** | 765±117 | 56.5±13.3 | 2.5±0.7 | 4 |
| **VA-TET** | 1091±87 | 34.1±6.3 | 1.0±0.1 | 5 |

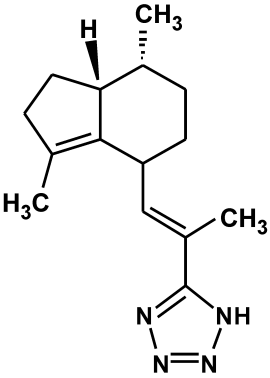
## α1β3γ2S

| | $E_{max}$ (%) | $EC_{50}$ (μM) | $n_H$ | n |
|---|---|---|---|---|
| **VA** | 632±88 | 20.2±5.2 | 1.5±0.3 | 9 |
| **VA-A** | 972±69 | 7.5±1.8 | 1.5±0.2 | 6 |
| **VA-MA** | 1043±57 | 12.7±0.9 | 1.5±0.1 | 6 |
| **VA-DMA** | 415±61 | 48.0±16.0 | 1.4±0.2 | 6 |
| **VA-EA** | 677±64 | 27.2±6.7 | 1.3±0.2 | 12 |
| **VA-DEA** | 374±102 | 80.8±31.3 | 1.9±0.5 | 5 |
| **VA-CN** | 522±114 | 42.4±15.8 | 2.1±0.8 | 7 |
| **VA-TET** | 668±57 | 6.0±1.0 | 1.1±0.1 | 8 |

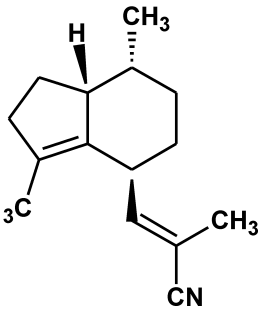
# Figure 1



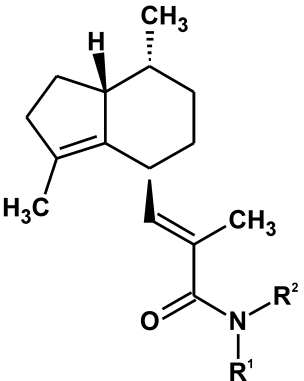

VA




VA-TET

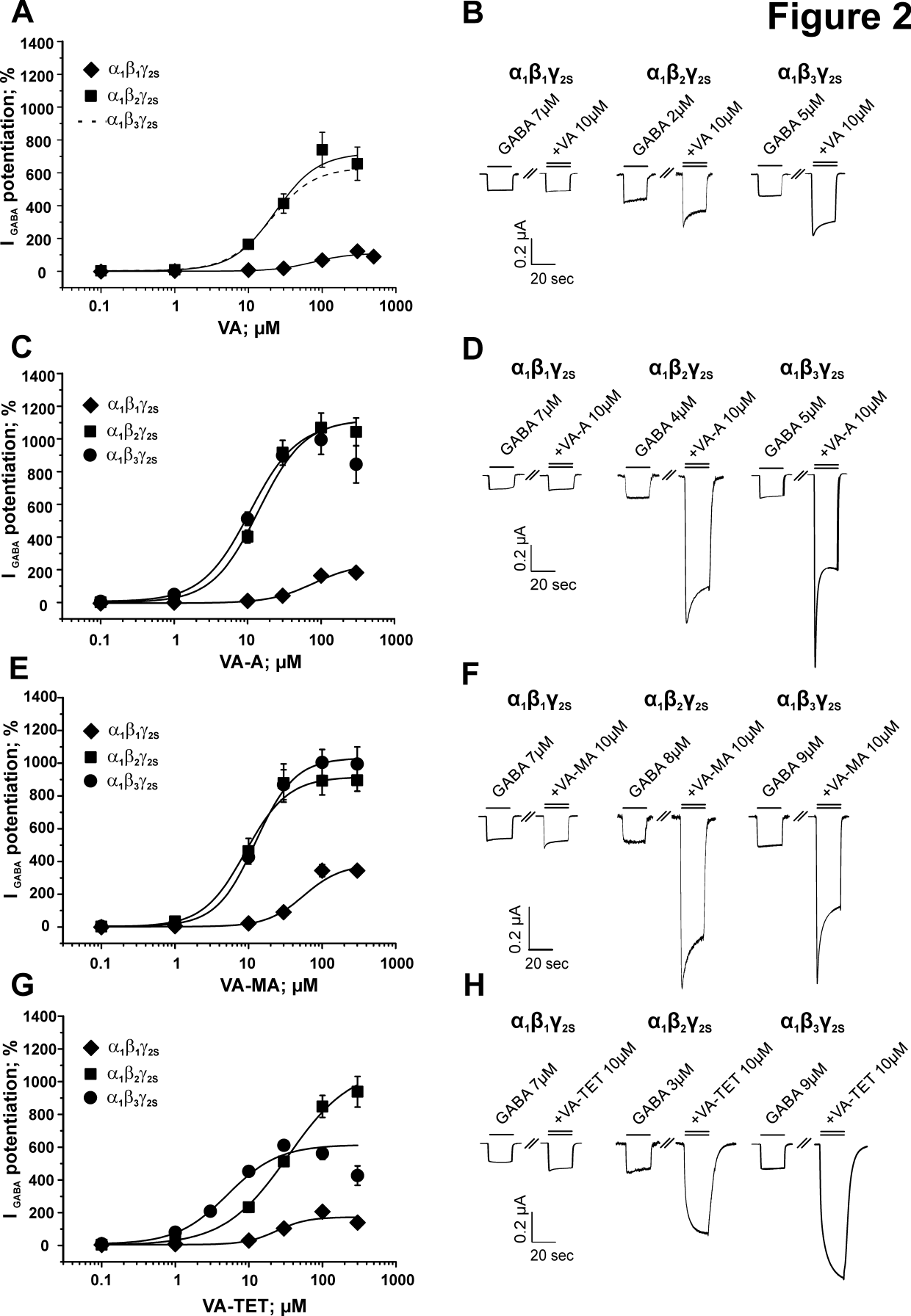



VA-CN

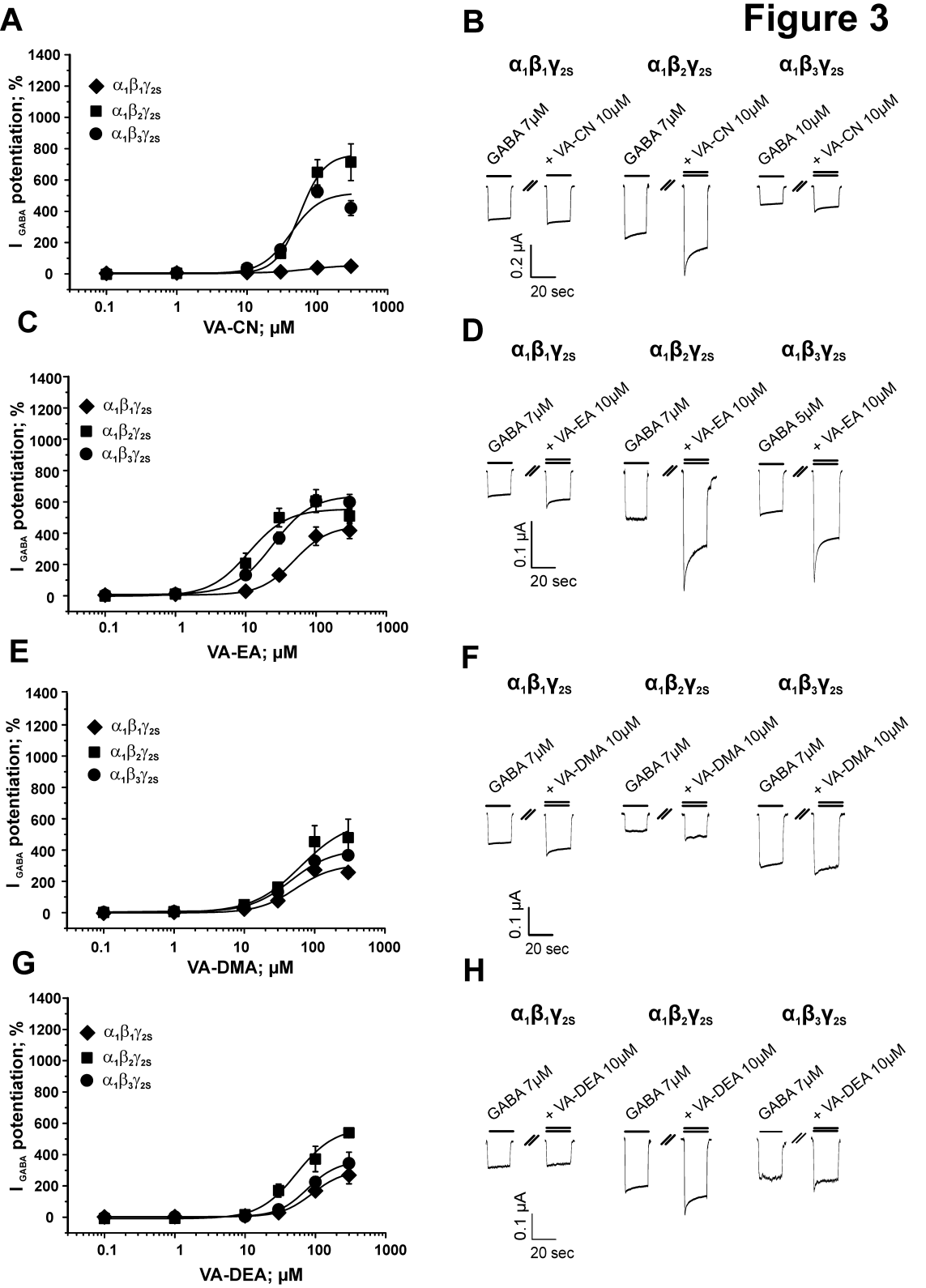



| Code | $R^1$ | $R^2$ |
|---|---|---|
| VA-A | H | H |
| VA-MA | $CH_3$ | H |
| VA-DMA | $CH_3$ | $CH_3$ |
| VA-EA | $CH_2$-$CH_3$ | H |
| VA-DEA | $CH_2$-$CH_3$ | $CH_2$-$CH_3$ |

# Figure 2


A
B
C
D
E
F
G
H
$\alpha_1\beta_1\gamma_{2S}$
$\alpha_1\beta_2\gamma_{2S}$
$\alpha_1\beta_3\gamma_{2S}$
$I_{GABA}$ potentiation; %
VA; μM
VA-A; μM
VA-MA; μM
VA-TET; μM
GABA 7μM
+VA 10μM
GABA 2μM
GABA 5μM
+VA-A 10μM
GABA 4μM
GABA 5μM
+VA-MA 10μM
GABA 8μM
GABA 9μM
+VA-TET 10μM
GABA 3μM
GABA 9μM
0.2 μA
20 sec

# Figure 3


A
B
C
D
E
F
G
H
$\alpha_1\beta_1\gamma_{2S}$
$\alpha_1\beta_2\gamma_{2S}$
$\alpha_1\beta_3\gamma_{2S}$
$I_{GABA}$ potentiation; %
VA-CN; μM
VA-EA; μM
VA-DMA; μM
VA-DEA; μM
GABA 7μM
+ VA-CN 10μM
GABA 10μM
+ VA-EA 10μM
GABA 5μM
+ VA-DMA 10μM
+ VA-DEA 10μM
0.2 μA
0.1 μA
20 sec

# Figure 4

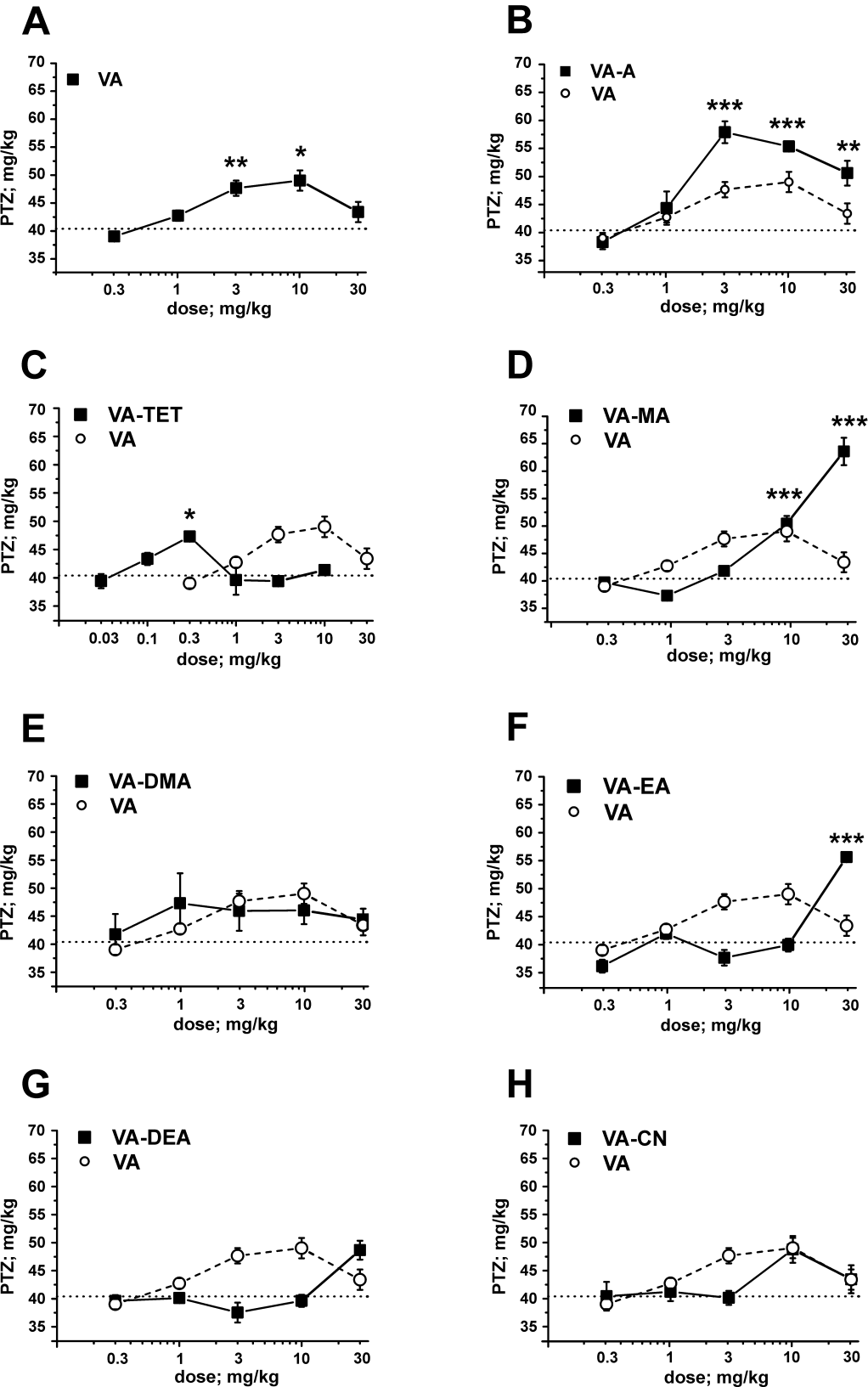
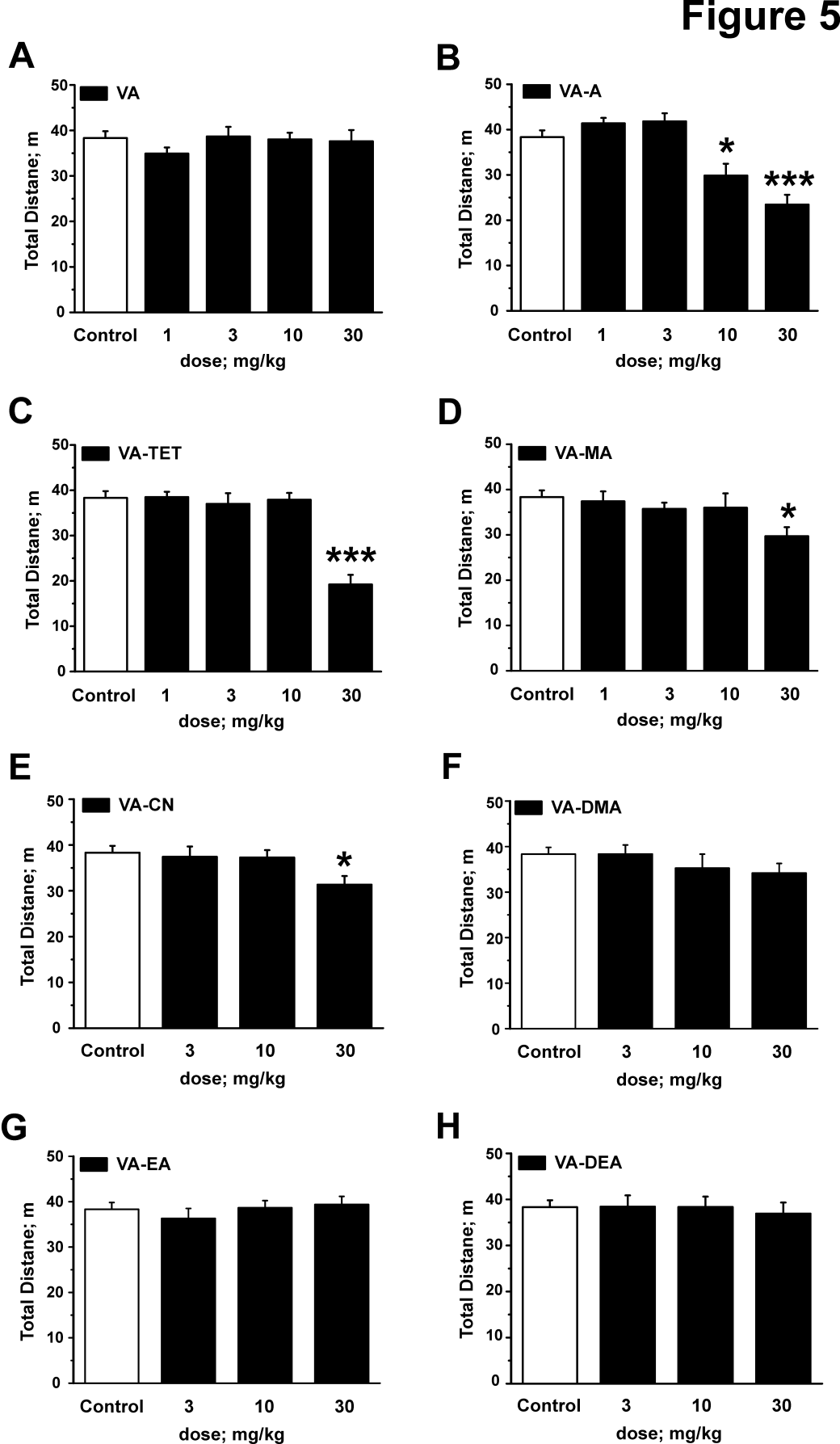

A
VA
PTZ; mg/kg
dose; mg/kg
B
VA-A
VA
C
VA-TET
VA
D
VA-MA
VA
E
VA-DMA
VA
F
VA-EA
VA
G
VA-DEA
VA
H
VA-CN
VA

Figure 5


A
VA
Total Distane; m
50
40
30
20
10
0
Control
1
3
10
30
dose; mg/kg
B
VA-A
Total Distane; m
*
***
Control
1
3
10
30
dose; mg/kg
C
VA-TET
Total Distane; m
***
Control
1
3
10
30
dose; mg/kg
D
VA-MA
Total Distane; m
*
Control
1
3
10
30
dose; mg/kg
E
VA-CN
Total Distane; m
*
Control
3
10
30
dose; mg/kg
F
VA-DMA
Total Distane; m
Control
3
10
30
dose; mg/kg
G
VA-EA
Total Distane; m
Control
3
10
30
dose; mg/kg
H
VA-DEA
Total Distane; m
Control
3
10
30
dose; mg/kg