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RESEARCH PAPER

Valerenic acid derivatives as novel subunit-selective $GABA_A$ receptor ligands – *in vitro* and *in vivo* characterization

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BACKGROUND AND PURPOSE

Subunit-specific modulators of γ-aminobutyric acid (GABA) type A ($GABA_A$) receptors can help to assess the physiological function of receptors with different subunit composition and also provide the basis for the development of new drugs. Valerenic acid (VA) was recently identified as a $\beta_{2/3}$ subunit-specific modulator of $GABA_A$ receptors with anxiolytic potential. The aim of the present study was to generate VA derivatives as novel $GABA_A$ receptor modulators and to gain insight into the structure–activity relation of this molecule.

EXPERIMENTAL APPROACH

The carboxyl group of VA was substituted by an uncharged amide or amides with different chain length. Modulation of $GABA_A$ receptors composed of different subunit compositions by the VA derivatives was studied in *Xenopus* oocytes by means of the two-microelectrode voltage-clamp technique. Half-maximal stimulation of GABA-induced chloride currents (I_{GABA}) through $GABA_A$ receptors (EC_{50}) and efficacies (maximal stimulation of I_{GABA}) were estimated. Anxiolytic activity of the VA derivatives was studied in mice, applying the elevated plus maze test.

KEY RESULTS

Valerenic acid amide (VA-A) displayed the highest efficacy (more than twofold greater I_{GABA} enhancement than VA) and highest potency (EC_{50} = 13.7 ± 2.3 μM) on $\alpha_1\beta_3$ receptors. Higher efficacy and potency of VA-A were also observed on $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_3\beta_3\gamma_{2s}$ receptors. Anxiolytic effects were most pronounced for VA-A.

CONCLUSIONS AND IMPLICATIONS

Valerenic acid derivatives with higher efficacy and affinity can be generated. Greater *in vitro* action of the amide derivative correlated with a more pronounced anxiolytic effect *in vivo*. The data give further confidence in targeting β_3 subunit containing $GABA_A$ receptors for development of anxiolytics.

Abbreviations

I_{GABA}, GABA-induced chloride currents; VA, valerenic acid; VA-A, valerenic acid amide; VA-BA, valerenic acid butylamide; VA-DEA, valerenic acid diethylamide; VA-DMA, valerenic acid dimethylamide; VA-EA, valerenic acid ethylamide; VA-EE, valerenic acid ethyl ester; VA-IPA, valerenic acid isopropylamide; VA-MA, valerenic acid methylamide; VA-MO, valerenic acid morpholine amide; VA-PIP, valerenic acid piperidine amide

Introduction

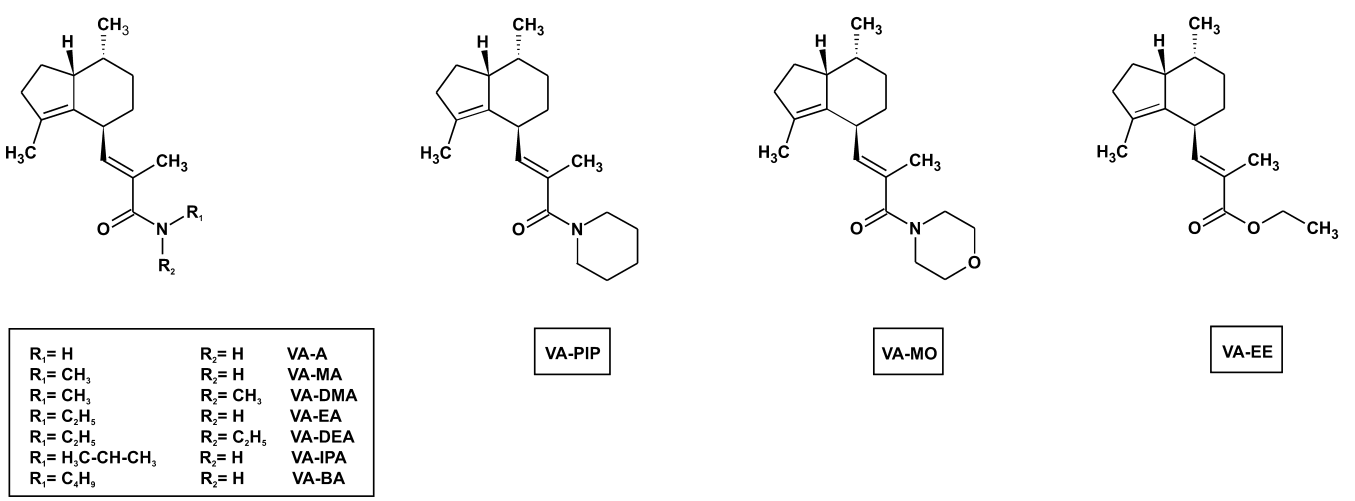
γ-Aminobutyric acid (GABA) mediates fast synaptic, inhibitory neurotransmission in the mammalian brain acting on GABA type A ($GABA_A$) receptors. $GABA_A$ receptors represent ligand gated chloride channels, assembled from different subunits forming a pentameric structure. Nineteen subunits of mammalian $GABA_A$ receptors have been cloned: α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, ρ_{1-3} and θ (Barnard *et al.*, 1998; Simon *et al.*, 2004; Olsen and Sieghart, 2008; receptor nomenclature follows Alexander *et al.*, 2009). The distribution of these subunits in the brain is highly distinct, suggesting differential functional roles for different $GABA_A$ receptors. The subunit composition determines the GABA sensitivity and the pharmacological properties of the $GABA_A$ receptor (Sieghart, 1995; D'Hulst *et al.*, 2009; Olsen and Sieghart, 2009).

γ-Aminobutyric acid type A receptors play a major role in controlling the excitability of the CNS. A disturbance of the balance between excitatory and inhibitory neurotransmission is associated with neurological disorders such as insomnia, anxiety disorders, epilepsy and schizophrenia (Sieghart and Sperk, 2002, Möhler, 2006a). Consequently, $GABA_A$ receptors represent the molecular target of many clinically important drugs such as benzodiazepines, barbiturates, general anaesthetics or the anticonvulsant loreclezole (see Sieghart 2006, Möhler, 2006b; Olsen and Sieghart, 2008). $GABA_A$ receptors are also modulated by natural compounds of plant origin (see Johnston *et al.*, 2006).

We have recently identified valerenic acid (VA), a major constituent of *Valeriana officinalis* L., as a subunit-specific modulator of $GABA_A$ receptors interacting exclusively with $GABA_A$ receptors comprising $\beta_{2/3}$ subunits, with no significant effect on GABA-induced chloride currents (I_{GABA}) through $GABA_A$ receptors containing β_1 subunits (Khom *et al.*, 2007). Moreover, enhancement of inhibitory, GABAergic neurotransmission by VA reduces anxiety-related behaviour *in vivo* (Benke *et al.*, 2009). Based on this subtype selectivity *in vitro* and anxiolytic effects *in vivo*, VA and its derivatives represent interesting drug candidates. In order to gain insight into the structure–activity relationship of these derivatives, we have synthesized 10 VA derivatives (Figure 1) and analysed their *in vitro* and *in vivo* effects. VA was converted into various amides with different lipophilicity and bulkiness. VA ethyl ester (VA-EE) emerged from the total synthesis described by Ramharter and Mulzer (2009).

The modulation of I_{GABA} by these VA derivatives through $GABA_A$ receptors expressed in *Xenopus* oocytes was analysed by means of the two-microelectrode voltage-clamp technique and a fast perfusion system. The behavioural effects of selected compounds were studied in the elevated plus maze test that allowed the identification of anxiolytic and sedative effects.

In vitro, short-chain VA amides such as VA amide (VA-A); VA methylamide (VA-MA) and VA ethylamide (VA-EA) revealed a stronger potentiation of I_{GABA} compared with VA itself, whereas side chain prolongation resulted in derivatives exhibiting a



R_1= H	R_2= H	VA-A
R_1= CH_3	R_2= H	VA-MA
R_1= CH_3	R_2= CH_3	VA-DMA
R_1= C_2H_5	R_2= H	VA-EA
R_1= C_2H_5	R_2= C_2H_5	VA-DEA
R_1= H_3C-CH-CH_3	R_2= H	VA-IPA
R_1= C_4H_9	R_2= H	VA-BA

Figure 1

Chemical structures of VA derivatives. VA-A, valerenic acid amide; VA-MA, valerenic acid methlyamide; VA-DMA, valerenic acid dimethylamide; VA-EA, valerenic acid ethylamide; VA-DEA, valerenic acid diethylamide; VA-IPA, valerenic acid isopropylamide; VA-BA, valerenic acid butylamide; VA-PIP, valerenic acid piperidine amide; VA-MO, valerenic acid morpholine amide; VA-EE, valerenic acid ethylate.

comparable or weaker enhancement of I_{GABA}. VA-A was more potent than VA *in vitro* and displayed the greatest anxiolytic effect *in vivo*.

Methods

Synthesis of VA derivatives

All reactions were carried out in oven-dried glassware under an argon atmosphere. Anhydrous CH_2Cl_2 was distilled under argon from P_2O_5. Commercially available reagents (purchased from Sigma, Vienna, Austria or Acros Organics, Vienna, Austria) were used without further purification. Reaction mixtures were magnetically stirred and monitored by thin layer chromatography with silica gel 60 F_{254} plates (E. Merck, Darmstadt, Germany). Flash column chromatography was performed with Merck silica gel (0.04–0.063 mm, 240–400 mesh) under pressure. Yields refer to chromatographically and spectroscopically pure compounds. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were either recorded on Bruker Avance 400, or DRX 400 spectrometers. All NMR spectra were measured in $CDCl_3$ solutions. The chemical shifts δ are reported relative to the residual solvent peaks. All ^{1}H and ^{13}C shifts are given in ppm (s = singlet; d = doublet; t = triplet; q = quadruplet; m = multiplet). If possible, assignments of proton resonances were confirmed by correlated spectroscopy. Optical rotations were measured at 20°C on a P 341 Perkin-Elmer polarimeter. IR spectra were recorded of samples prepared as films on silicium plates on a Perkin-Elmer Spectrum 1600 Series FTIR spectrometer. MS spectra were measured on a Finnigan MAT 8230 apparatus with a resolution of 10 000.

VA-A

Valerenic acid (104.3 mg, 0.4451 mM) was dissolved in anhydrous CH_2Cl_2 (4.4 mL) cooled to 0°C and 1-chloro-N,N,2-trimethylpropenylamine (0.21 mL, 212.1 mg, 1.587 mM) was added dropwise. After 5 min the icebath was removed and the reaction mixture stirred for 4 h at room temperature before being re-cooled to 0°C. Ammonia (0.5 M) in 1,4-dioxane (5.8 mL, 2.900 mM) was rapidly added, the icebath removed and the resulting mixture stirred overnight. Diethylether was added and the white precipitate removed by filtration. The filtrate was concentrated *in vacuo* and chromatography (hexane/EtOAc 2/1 to 1/2) of the residue provided 99.8 mg of VA-A as a white solid with 96% yield.

^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 6.67 (dq, 1 H, J = 0.8 Hz, J = 9.3 Hz), 5.58 (s, 2 H), 3.50 (dd, 1 H, J = 4.7 Hz, J = 9.4 Hz), 2.93 (m, 1 H), 2.19 (t, 2 H, J = 7.5 Hz), 1.98 (m, 1 H), 1.90 (d, 3 H, J = 3.3 Hz), 1.78 (m, 3 H), 1.63 (m, 3 H), 1.55 (m, 1 H), 1.40 (m, 2 H), 0.77 (d, 3 H, J = 7.0 Hz)

^{13}C-NMR (100 MHz, $CDCl_3$, ppm): 171.8, 139.1, 133.7, 130.9, 128.3, 47.6, 37.6, 34.3, 33.2, 28.9, 25.7, 24.7, 13.6, 12.9, 12.2

IR (film, cm^{-1}): 3179, 3028, 2923, 2885, 2853, 1733, 1654, 1652, 1636, 1505, 1458, 1418, 1378

HRMS (ESI, m/z): $[M]^+$ calc. 215.1674; found: 215.1661

$[\alpha]_D^{20}$ = –119.2 (c = 0.775 g per 100 mL, DCM)

VA-MA

Using the same procedure as for the preparation of VA-A, VA (49.7 mg, 0.2121 mM) was treated with 1-chloro-N,N,2-trimethylpropenylamine (0.098 mL, 99.0 mg, 0.7408 mM) and methylamine (41% aqueous solution, 0.18 mL, 2.131 mM) to afford 49.9 mg of VA-MA as a white solid with 95% yield.

^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 6.54 (dq, 1 H, J = 1.3 Hz, J = 9.5 Hz), 5.71 (s, 1 H), 3.48 (dd, 1 H, J = 4.7 Hz, J = 9.2 Hz), 2.93 (m, 1 H), 2.86 (d, 3 H, J = 4.9 Hz), 2.19 (t, 2 H, J = 7.3 Hz), 1.97 (m, 1 H), 1.89 (d, 3 H, J = 1.4 Hz), 1.78 (m, 3 H), 1.62 (m, 3 H), 1.55 (m, 1 H), 1.39 (m, 2 H), 0.77 (d, 3 H, J = 7.0 Hz)

^{13}C-NMR (100 MHz, $CDCl_3$, ppm): 170.7, 137.0, 134.0, 130.7, 129.5, 47.6, 37.6, 34.1, 33.3, 28.9, 26.7, 25.8, 24.7, 13.6, 12.9, 12.2

IR (film, cm^{-1}): 3326, 2925, 1655, 1616, 1539, 1456, 1379, 1311, 670, 667, 409

HRMS (ESI, m/z): $[M]^+$ calc. 247.1936; found: 247.1932

$[\alpha]_D^{20}$ = –129.3 (c = 0.825 g per 100 mL, DCM)

VA-EA

Using the same procedure as for the preparation of VA-A, VA (20.9 mg, 0.0892 mM) was treated with 1-chloro-N,N,2-trimethylpropenylamine (0.035 mL, 35.4 mg, 0.2646 mM) and 2.0 M ethylamine in THF (0.45 mL, 0.9000 mM) to afford 21.9 mg of VA EA as a slightly yellow solid with 94% yield.

^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 6.55 (dq, 1 H, J = 1.3 Hz, J = 9.5 Hz), 5.66 (s, 1 H), 3.49 (dd, 1 H, J = 4.9 Hz, J = 9.3 Hz), 3.34 (dq, 2 H, J = 5.6 Hz, J = 7.2 Hz), 2.95 (m, 1 H), 2.19 (t, 2 H, J = 7.5 Hz), 1.98 (m, 1 H), 1.89 (d, 3 H, J = 1.4 Hz), 1.78 (m, 3 H), 1.62 (m, 3 H), 1.54 (m, 1 H), 1.40 (m, 2 H), 1.17 (t, 3 H, J = 7.3 Hz), 0.78 (d, 3 H, J = 7.0 Hz)

^{13}C-NMR (100 MHz, $CDCl_3$, ppm): 169.9, 136.9, 134.0, 130.6, 129.6, 47.6, 37.6, 34.8, 34.1, 33.3, 28.9, 25.8, 24.7, 15.1, 13.6, 12.9, 12.2

IR (film, cm^{-1}): 3308, 2925, 2884, 2855, 1653, 1639, 1616, 1533, 1456, 1379, 1309

HRMS (ESI, m/z): $[M]^+$ calc. 262.2171; found: 262.2161

$[\alpha]_D^{20}$ = –118.7 (c = 1.58 g per 100 mL, DCM)

Valerenic N,N-diethylamide (VA-DEA)

Using the same procedure as for the preparation of VA-A, VA (30.2 mg, 0.1289 mM) was treated with 1-chloro-N,N,2-trimethylpropenylamine (0.051 mL, 51.5 mg, 0.3855 mM) and diethylamine (0.135 mL, 95.9 mg, 1.311 mM) to afford 35.2 mg of VA-DEA as a slightly yellow oil with 94% yield.

^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 5.75 (dq, 1 H, J = 0.5 Hz, J = 7.6 Hz), 3.46 (dd, 1 H, J = 3.9 Hz, J = 8.5 Hz), 3.34 (m, 4 H), 2.85 (m, 1 H), 2.17 (t, 2 H, J = 7.4 Hz), 1.96 (m, 1 H), 1.87 (d, 3 H, J = 1.5 Hz), 1.77 (m, 3 H), 1.63 (m, 3 H), 1.52 (m, 1 H), 1.41 (m, 2 H), 1.13 (t, 6 H, J = 7.1 Hz), 0.77 (d, 3 H, J = 7.0 Hz)

^{13}C-NMR (100 MHz, $CDCl_3$, ppm): 173.8, 134.3, 131.0, 130.3, 130.1, 47.7, 37.6, 33.5, 33.3, 29.0, 25.8, 24.8, 14.6, 13.6, 12.2

IR (film, cm^{-1}): 2926, 2866, 2855, 1628, 1458, 1396, 1380, 1333, 1291, 1221,1097

HRMS (ESI, m/z): $[M]^+$ calc. 290,2484; found: 290,2488

$[\alpha]_D^{20}$ = –109.8 (c = 1.63 g per 100 mL, DCM)

Valerenic acid morpholine amide (VA-MO)

Using the same procedure as for the preparation of VA-A, VA (43.4 mg, 0.1852 mM) was treated with 1-chloro-N,N,2-trimethylpropenylamine (0.074 mL, 74.7 mg, 0.5593 mM) and morpholine (0.160 mL, 160.0 mg, 1.837 mM) to afford 50.4 mg of VA-MO as a colourless oil with 90% yield.

^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 5.82 (dq, 1 H, J = 1.5 Hz, J = 9.1 Hz), 3.66 (t, 4 H, J = 4.5), 3.56 (m, 4 H), 3.47 (dd, 1 H, J = 2.8 Hz, J = 9.0 Hz), 2.82 (m, 1 H), 2.18 (t, 2 H, J = 7.4 Hz), 1.97 (m, 1 H), 1.87 (d, 3 H, J = 1.5 Hz), 1.78 (m, 3 H), 1.64 (m, 3 H), 1.55 (m, 1 H), 1.41 (m, 2 H), 0.77 (d, 3 H, J = 7.0 Hz)

^{13}C-NMR (100 MHz, $CDCl_3$, ppm): 172.9, 134.0, 133.6, 130.4, 128.9, 67.1, 47.8, 37.6, 33.6, 33.4, 29.0, 25.9, 24.8, 14.5, 13.6, 12.2

IR (film, cm^{-1}): 2923, 1654, 1652, 1628, 1420, 1393, 1379, 1245, 1116, 1028, 842

HRMS (ESI, m/z): $[M]^+$ calc. 303.2198; found: 303.2194

$[\alpha]_D^{20}$ = –110.5 (c = 0.8 g per 100 mL, DCM)

Valerenic N,N-dimethylamide (VA-DMA)

A solution of VA (117 mg, 0.5 mM) in anhydrous CH_2Cl_2 (5 mL) was cooled to 0°C and 1-chloro-N,N,2-trimethylpropenylamine (0.67 mL, 2 mM) was added dropwise. After 5 min the icebath was removed and the reaction mixture stirred for 4 h at room temperature. Then the solvent and volatile by-products were removed *in vacuo* (3 Torr for 1 h), and the crude acid chloride was redissolved in CH_2Cl_2 (5 mL). After cooling to 0°C, dimethylamine (0.51 mL, 5 mM) was added at once, the icebath removed and the resulting mixture stirred for 1 h. Diethylether was added and the white precipitate removed by filtration. The filtrate was concentrated *in vacuo* and chromatography (toluol/EtOAc 3/2) of the residue provided 111 mg of VA-DMA as a slightly yellow oil with 85% yield.

^{1}H-NMR (200 MHz, CDCl3, ppm): 5.83 (dd, 1 H, J = 1.5 Hz, J = 9.1 Hz), 3.48 (m, 1 H), 2.98 (s, 6 H), 2.86 (m, 1 H), 2.20 (t, 2 H, J = 7.2 Hz), 1.87 (m, 7 H), 1.49 (m, 5 H), 0.77 (dd, 3 H, J = 6.9 Hz)

^{13}C-NMR (50 MHz, CDCl3, ppm): 174.0, 134.0, 132.8, 129.9, 129.5, 47.5, 37.4, 33.3, 33.2, 28.8, 25.7, 24.6, 14.1, 13.4, 12.0

IR (film, cm^{-1}): 2926, 1627, 1496, 1448, 1390, 1091

HRMS (ESI, m/z): $[M]^+$ calc. 262.2171 found: 262.2189

$[\alpha]_D^{20}$ = –104.3 (c = 0.54 g per 100 mL, DCM)

Valerenic acid piperidine amide (VA-PIP)

A solution of VA (43.5 mg, 0.1856 mM) in anhydrous CH_2Cl_2 (1.8 mL) was cooled to 0°C and 1-chloro-N,N,2-trimethylpropenylamine (0.086 mL, 86.9 mg, 0.6500 mM) was added dropwise. After 5 min the icebath was removed and the reaction mixture stirred for 4 h at room temperature. Then the solvent and volatile by-products were removed *in vacuo* (3 Torr for 1 h), and the crude acid chloride was redissolved in CH_2Cl_2 (1.8 mL). After cooling to 0°C, piperidine (0.074 mL, 63.6 mg, 0.7474 mM) was added at once, the icebath removed and the resulting mixture stirred for 1 h. Diethylether was added and the white precipitate removed by filtration. The filtrate was concentrated *in vacuo* and chromatography (hexane/EtOAc 5/1 to 2/1) of the residue provided 50.7 mg of VA-PIP as a slightly yellow oil with 91% yield.

^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 5.78 (dq, 1 H, J = 1.5 Hz, J = 9.1 Hz), 3.45 (m, 5 H), 2.84 (m, 1 H), 2.18 (t, 2 H, J = 4.9 Hz), 1.96 (m, 1 H), 1.85 (d, 3 H, J = 1.5 Hz), 1.77 (m, 3 H), 1.63 (m, 5 H), 1.54 (m, 5 H), 1.40 (m, 2 H), 0.77 (d, 3 H, J = 7.0 Hz)

^{13}C-NMR (100 MHz, $CDCl_3$, ppm): 172.8, 134.3, 131.9, 130.0, 129.8, 47.7, 37.6, 33.5, 33.4, 29.0, 25.9, 24.9, 24.8, 14.5, 13.6, 12.2

IR (film, cm^{-1}): 2925, 2854, 1629, 1443, 1379, 1256, 425, 423, 420,412

HRMS (ESI, m/z): $[M]^+$ calc. 301.2406; found: 301.2398

$[\alpha]_D^{20}$ = –90.0 (c = 0.35 g per 100 mL, DCM)

Valerenic N-isopropylamide (VA-IPA)

A solution of VA (117 mg, 0.5 mM) in anhydrous CH_2Cl_2 (5 mL) was cooled to 0°C, and 1-chloro-N,N,2-trimethylpropenylamine (0.2 mL, 0.6 mM) was added dropwise. After 5 min the icebath was removed and the reaction mixture stirred for 4 h at room temperature. Then the solvent and volatile by-products were removed *in vacuo* (3 Torr for 1 h) and the crude acid chloride was redissolved in CH_2Cl_2 (5 mL). After cooling to 0°C n-isopropylamine (0.43 mL, 5 mM) was added at once, the icebath removed and the resulting mixture stirred for 1 h. Diethylether was added and the white precipitate removed by filtration. The filtrate was concentrated *in vacuo* and chromatography (toluole/EtOAc 3/2) of the residue provided 108 mg of VA-IPA as slightly yellow crystals with 78% yield.

^{1}H-NMR (200 MHz, CDCl3, ppm): 6.55 (dd, 1 H, J = 1.3 Hz, J = 9.5 Hz), 5.53 (m, 1 H), 4.12 (m, 1 H), 3.49 (m, 1 H), 2.95 (m, 1 H), 2.20 (t, 2 H, J = 7.4), 1.75 (m, 11 H), 1.39 (m, 2 H), 1.18 (d, 6 H, J = 6.6 Hz), 0.77 (d, 3 H, J = 6.9 Hz)

^{13}C-NMR (50 MHz, CDCl3, ppm): 168.9, 136.6, 133.8, 130.4, 129.4, 47.4, 41.4, 37.4, 33.9, 33.1, 28.7, 25.6, 24.5, 22.8, 13.4, 12.7, 12.0

IR (film, cm^{-1}): 3306, 2930, 1652, 1611, 1533, 1456

HRMS (ESI, m/z): [M]+ calc. 276.2327; found: 276.2332

$[\alpha]_D^{20}$ = –126.6 (c = 0.50 g per 100 mL, DCM)

Valerenic N-butylamide (VA-BA)

A solution of VA (117 mg, 0.5 mM) in anhydrous CH_2Cl_2 (5 mL) was cooled to 0°C and 1-chloro-N,N,2-trimethylpropenylamine (0.2 mL, 0.6 mM) was added dropwise. After 5 min the icebath was removed and the reaction mixture stirred for 4 h at room temperature. Then the solvent and volatile by-products were removed *in vacuo* (3 Torr for 1 h), and the crude acid chloride was redissolved in CH_2Cl_2 (5 mL). After cooling to 0°C, n-butylamine (0.28 mL, 5 mM) was added at once, the icebath removed and the resulting mixture stirred for 1 h. Diethylether was added and the white precipitate removed by filtration. The filtrate was concentrated *in vacuo* and chromatography (toluole/EtOAc 3/2) of the residue provided 102 mg of VA-BA as slightly yellow crystals with 70% yield.

^{1}H-NMR (200 MHz, CDCl3, ppm): 6.55 (dd, 1 H, J = 1.3 Hz, J = 9.3 Hz), 5.72 (s, 1 H), 3.48 (m, 1 H), 3.29 (q, 2 H, J = 6.82), 2.93 (m, 1 H), 1.86 (t, 2 H, J = 7.3), 1.86 (m, 7 H), 1.49 (m, 10 H), 0.93 (t, 3 H, J = 7.2), 0.77 (d, 3 H, J = 6.9)

^{13}C-NMR (50 MHz, CDCl3, ppm): 169.7, 136.7, 133.8, 130.4, 129.3, 47.4, 39.5, 37.4, 33.9, 33.1, 31.8, 28.7, 25.6, 24.5, 20.1, 13.8, 13.4, 12.8, 12.0

IR (film, cm^{-1}): 3306, 2929, 1652, 1616, 1538, 1380

HRMS (ESI, m/z): [M]$^+$ calc. 290.2484; found: 290.2488

$[\alpha]_D^{20}$ = –121.5 (c = 0.47 g per 100 mL, DCM)

Molecular modelling studies

Molecules used for the Free-Wilson analysis were built in MOE (version 2009.10) and energy minimized using standard conditions (MMFF94x force field, adjust H and LP, gradient = 0.01, calculate force field partial charges). A database was built and logP(o/w), TPSA and mr were calculated as descriptors. Correlation analyses were performed with the correlation plot tool implemented in MOE.

Pharmacological characterization of VA derivatives

For *in vitro* and *in vivo* experiments, stock solutions (100 mM) were prepared in dimethyl sulphoxide (DMSO; Sigma, Vienna, Austria). In voltage-clamp 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.*, 2007). For *in vivo* experiments, working concentrations were adjusted by dilution with 0.9% sodium chloride. pH was adjusted to 7.2–7.4 with NaOH. 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*, synthesis of capped off run-off poly(A$^+$) cRNA transcripts from linearized cDNA templates (pCMV vector) was performed as described (Khom *et al.*, 2006). Briefly, female *X. laevis* (NASCO, USA) were anaesthetized by exposing them for 15 min to a 0.2% solution of MS-222 (methane sulphonate salt of 3-aminobenzoic acid ethyl ester; Sigma) before surgically removing parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg·mL^{-1} collagenase (Type 1A, Sigma). One day after isolation, the oocytes were injected with about 10–50 nL of DEPC-treated water (diethyl pyrocarbonate, Sigma) containing the different cRNAs at a concentration of approximately 150–3000 ng·µL^{-1}·subunit^{-1}. The amount of cRNA was determined by means of a NanoDrop ND-1000 (Kisker-biotech, Steinfurt, Germany).

To ensure expression of the gamma-subunit in the case of $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_3\beta_3\gamma_{2S}$ receptors, cRNAs were mixed in a ratio of 1:1:10 and for receptors comprising only α- and β-subunits ($\alpha_1\beta_2$, $\alpha_1\beta_3$) in a ratio 1:1 (Khom *et al.*, 2007). cRNAs for $\alpha_1\beta_1$ channels were injected in a ratio of 3:1 to avoid formation of β_1

homo-oligomeric $GABA_A$ receptors (Krishek *et al.*, 1996). Oocytes were stored at 18°C in ND96 solution (Methfessel *et al.*, 1986). Electrophysiological experiments were done using the two-microelectrode voltage-clamp method 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 were acquired using pCLAMP v.9.2. The bath solution contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2$ and 5 mM HEPES (pH 7.4). Microelectrodes were filled with 2 M KCl and had resistances between 1 and 3 MΩ (Khom *et al.*, 2007).

Perfusion system

γ-Aminobutyric acid and VA derivatives were applied by means of a fast perfusion system (see Baburin *et al.*, 2006 for details). 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 a rate of 300–1000 $\mu L \cdot s^{-1}$. The I_{GABA} rise time ranged between 100 and 250 ms (Baburin *et al.*, 2006; Khom *et al.*, 2006). Care was taken to account for possible slow recovery from increasing levels of desensitization in the presence of high GABA and drug concentrations. The duration of washout periods was therefore extended from 1.5 min (1–20 µM GABA, <10 µM drug) to 30 min (≥100 µM GABA, ≥10 µM drug) respectively. Oocytes with maximal current amplitudes >3 µA were discarded to exclude voltage-clamp errors (Khom *et al.*, 2007).

Analysing concentration–response curves

Stimulation of chloride currents by modulators of the $GABA_A$ receptor was measured at a GABA concentration eliciting between 3% and 5% of the maximal current amplitude (EC_{3-5}). The EC_{3-5} was determined at the beginning of each experiment.

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 GABA and a given compound (VA or VA derivative), 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 1 min before applying GABA (EC_{3-5}). 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 ± SE from at least four oocytes and ≥2 oocyte batches. Statistical significance was calculated using paired Student's *t*-test with a confidence interval of $P < 0.05$.

Animals

All animal care and experimental procedures 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. Every effort was taken to minimize the number of animals used. Male mice (c57Bl/6N) were obtained from Charles River Laboratories (Sulzfeld, Germany). For breeding and maintenance, mice were group-housed with free access to food and water. Temperature (23 ± 1°C) and humidity (60%) were fixed and the animals housed with a 12 h light–dark cycle (lights on 0700–1900 h). Male mice at 3–6 months age were tested in all experiments.

Elevated plus maze test

Explorative behaviour was tested over 5 min on an elevated plus maze 1 m above ground. The maze consisted of two closed and two open arms, each 50 × 5 cm in size as previously described by Wittmann *et al.* (2009). The test instrument was built from gray PVC; the height of closed arm walls was 20 cm. Illumination was set to 180 lx. Animals were placed in the centre, facing an open arm. Open and closed arm entries and time spent on open arm was automatically analysed using Video-Mot 2 equipment and software (TSE, Bad Homburg, Germany).

Statistical analysis of behavioural experiments

For comparison of control groups and compound-treated groups, the unpaired Student's *t*-test was used. Comparison of more than two groups was done by one-way ANOVA. *P*-values of <0.05 were accepted as statistically significant. All data are given as mean ± SEM (*n*).

Materials

Valerenic acid was obtained from Extrasynthese (Lyon, France) and DMSO was purchased from Sigma. VA-EE was an intermediate of the total VA synthesis described by Ramharter and Mulzer (2009). The other tested derivatives were synthesized as described above.

Results

We have synthesized a series of novel VA derivatives (for structures, see Figure 1) to study the structure–

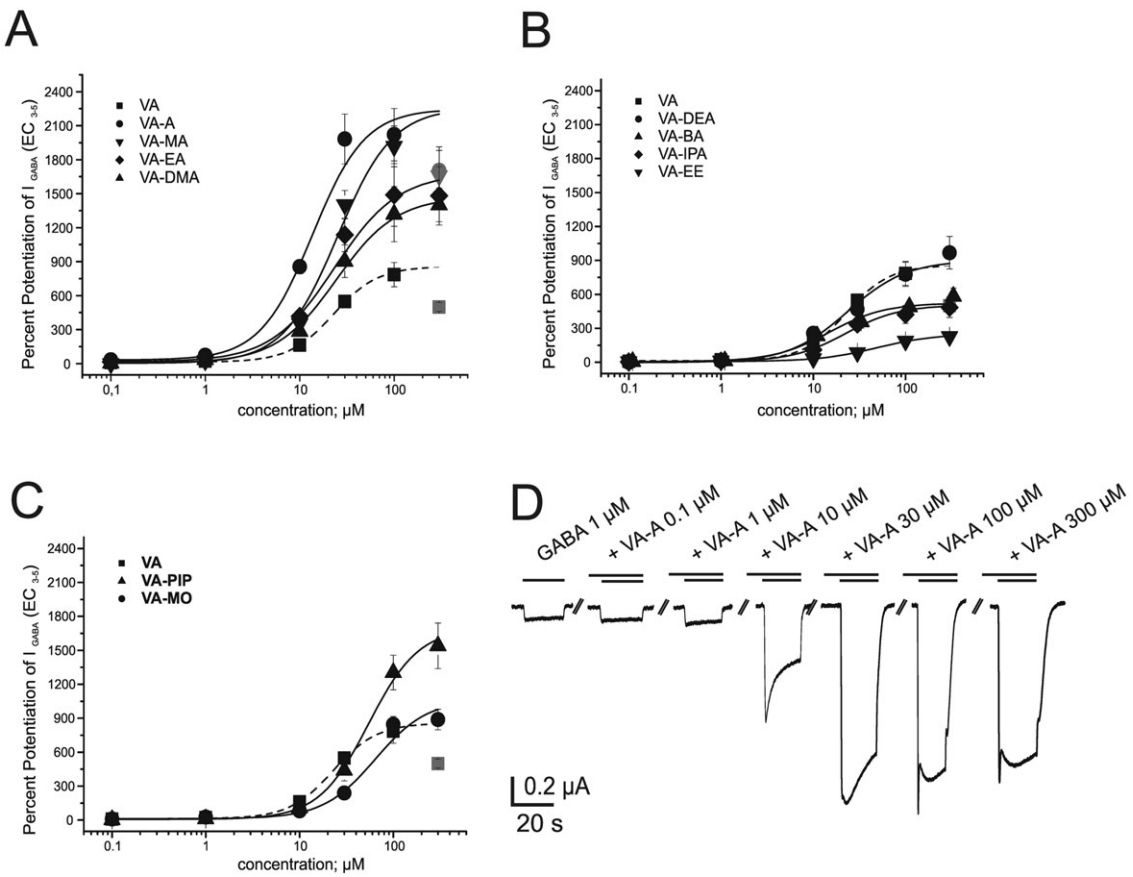


Figure 2

Concentration–effect curves for the enhancement of I_{GABA} through GABA$_A$ receptors composed of $\alpha_1\beta_3$ subunits by (A) VA, VA-A, VA-MA, VA-EA and VA-DMA; (B) VA, VA-DEA, VA-BA, VA-IPA and VA-EE; (C) VA, VA-PIP and VA-MO, using a GABA EC_{3-5} (EC_{50} and n_H values are given in Table 1). I_{GABA} at 300 µM (VA and VA-A) (Figure B) (grey symbols) were excluded from the fit. (D) Typical I_{GABA} recordings illustrating concentration-dependent modulation by VA-A of GABA elicited chloride currents through $\alpha_1\beta_3$ subunit-containing receptors. An open channel block at high VA-A concentrations was evident from the initial rapid current decay at 100 and 300 µM. GABA, γ-aminobutyric acid; I_{GABA}, GABA-induced chloride currents; VA, valerenic acid; VA-A, valerenic acid amide; VA-BA, valerenic acid butylamide; VA-DEA, valerenic acid diethylamide; VA-DMA, valerenic acid dimethylamide; VA-EA, valerenic acid ethylamide; VA-EE, valerenic acid ethyl ester; VA-IPA, valerenic acid isopropylamide; VA-MA, valerenic acid methylamide; VA-MO, valerenic acid morpholine amide; VA-PIP, valerenic acid piperidine amide.

activity relationship of these molecules on GABA$_A$ receptors composed of α_1 and β_3 subunits, expressed in *Xenopus* oocytes.

Modulation of I_{GABA} *by the VA derivatives*

We measured modulation of I_{GABA} by the VA derivatives by the two-microelectrode voltage-clamp technique. As shown in Figure 2A–C, all VA derivatives enhanced I_{GABA}. We observed, however, significant differences in efficacies and potencies.

Unsubstituted VA-A. VA-A was identified as the most effective and most potent VA derivative. Representative I_{GABA} modulated by VA-A are shown in Figure 2D. Relative to the parent compound, amidation resulted in an increased potency and a more than twofold enhanced maximal stimulation of I_{GABA} at GABA EC_{3-5} concentrations (see Figure 2A and Table 1).

Alkylated VA-amides. Introduction of aliphatic, alkyl residues resulted in a stronger modulation of I_{GABA} compared with VA. This effect was, however, less pronounced than for VA-A. Moreover, the nature of the alkyl residue significantly affected the efficacy of the amides: VA derivatives comprising residues such as monomethyl and monoethyl displayed a stronger I_{GABA} potentiation compared with VA. Bulkier residues such as dimethyl, diethyl, butyl and isopropyl, either decreased I_{GABA} enhancement or enhancement was not statistically different from VA (for details, see Figure 2 and Table 1).

Table 1

Summary of efficacies and potencies of tested VA derivatives

	EC_{50} (μM)	Maximum stimulation of I_{GABA} (EC_{3-5}) (%)	Hill coefficient (n_H)	Number of experiments (n)
VA	22.8 ± 5.6	858 ± 153	1.9 ± 0.3	5
VA-A	13.7 ± 2.3*	2247 ± 252*	1.6 ± 0.2	7
VA-MA	26.3 ± 6.6	2298 ± 312*	1.4 ± 0.1	6
VA-DMA	28.4 ± 7.1	1383 ± 211	2.1 ± 0.5	5
VA-EA	23.4 ± 6.9	1678 ± 258*	1.3 ± 0.3	5
VA-DEA	23.7 ± 6.3	901 ± 120	1.4 ± 0.1	6
VA-BA	18.8 ± 6.9	569 ± 57	1.1 ± 0.2	5
VA-IPA	22.5 ± 6.0	506 ± 76	1.5 ± 0.3	4
VA-PIP	54.6 ± 17.0*	1698 ± 266*	1.6 ± 0.3	6
VA-MO	64.2 ± 13.8*	1064 ± 132	1.6 ± 0.2	7
VA-EE	49.2 ± 26.3	250 ± 65*	1.4 ± 0.6	5

*$P < 0.05$, significantly different from corresponding values for VA.
GABA, γ-aminobutyric acid; I_{GABA}, GABA-induced chloride currents; VA, valerenic acid; VA-A, valerenic acid amide; VA-BA, valerenic acid butylamide; VA-DEA, valerenic acid diethylamide; VA-DMA, valerenic acid dimethylamide; VA-EA, valerenic acid ethylamide; VA-EE, valerenic acid ethyl ester; VA-IPA, valerenic acid isopropylamide; VA-MA, valerenic acid methylamide; VA-MO, valerenic acid morpholine amide; VA-PIP, valerenic acid piperidine amide.

Interestingly, introduction of cyclic residues on the amide nitrogen induced a significant loss of potency for VA-PIP and VA-MO (Table 1). Enhancement of I_{GABA} by VA-PIP was, however, comparable to that of VA-A, while potentiation of I_{GABA} by VA-MO was less pronounced, comparable to that of VA. VA-EE displayed the lowest efficacy and a reduced potency (Table 1).

β Subunit-specific modulation of I_{GABA} *by VA-A*

According to our previous study, VA selectively enhances I_{GABA} through $GABA_A$ receptors comprising either β_2 or β_3 subunits. Even high concentrations of VA (100 μM) induce no significant ($P < 0.05$) potentiation of I_{GABA} through receptors containing β_1 subunits. A similar study was performed with VA-A and I_{GABA} potentiation analysed for $GABA_A$ receptors composed of $\alpha_1\beta_1$, $\alpha_1\beta_2$ and $\alpha_1\beta_3$ subunits. VA-A displayed high selectivity for $\alpha_1\beta_2$ and $\alpha_1\beta_3$ receptors (Figure 3). Enhancement of I_{GABA} by VA-A was most pronounced through $GABA_A$ receptors composed of α_1 and β_3 subunits (maximal stimulation 2021 ± 231% at a concentration of 100 μM, EC_{50} = 13.7 ± 2.3 μM). VA-A also potentiated I_{GABA} through $GABA_A$ channels comprising β_2 subunits (maximal stimulation 1204 ± 270% at a concentration of 100 μM; EC_{50} = 8.2 ± 5.1 μM), but did not significantly enhance I_{GABA} through $\alpha_1\beta_1$ containing receptors even at 300 μM VA-A ($P < 0.05$).

Modulation of I_{GABA} *through* $\alpha_3\beta_3\gamma_{2S}$ *and* $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ *receptors by VA-A*

As shown in Figure 3, VA-A induced the most efficient I_{GABA} enhancement on receptors composed of $\alpha_1\beta_3$ receptors ($\alpha_1\beta_3 > \alpha_1\beta_2 >> \alpha_1\beta_1$, see similar data for VA in Khom *et al.*, 2007).

To gain insight into modulation of more physiological $GABA_A$ receptors (McKernan and Whiting, 1996; Olsen and Sieghart, 2008) we investigated the modulation of $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_3\beta_3\gamma_{2S}$ receptors by VA-A. As illustrated in Figure 4A, $\alpha_1\beta_2\gamma_{2S}$ receptors were more efficiently modulated by VA-A than by VA. Co-expression of a γ_{2S} subunit had no significant effects on efficacy or on potency ($P > 0.05$; compare Figures 3A and 4A, see Khom *et al.*, 2006 for comparable data obtained with VA). VA-A displayed also a higher efficacy than VA on receptors composed of $\alpha_3\beta_3\gamma_{2S}$ subunits (Figure 4B, $P < 0.05$, see also Table 2). On this receptor subtype, only a trend towards a higher potency was observed for VA-A, compared with VA (Tables 2, $P > 0.05$).

In vivo *effects of selected VA derivatives*

In order to get insights into the *in vivo* effects of VA derivatives, the effect of selected compounds on anxiety-related behaviour in mice was studied in the elevated plus maze test. Thirty minutes after i.p. injection of either solvent (=control) or drug containing solution (3 mg drug·kg^{-1}), the mice were tested for 5 min in the elevated plus maze. Mice treated with VA spent significantly more ($P < 0.05$)

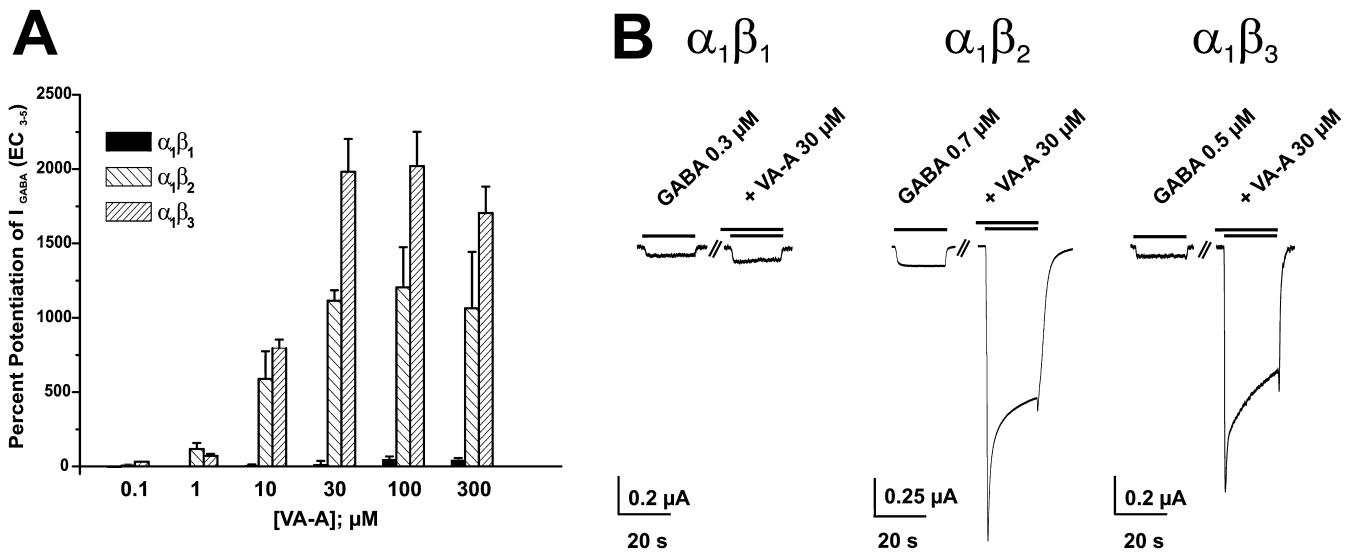


Figure 3

(A) Concentration-dependent effects for VA-A on $\alpha_1\beta_1$, $\alpha_1\beta_2$ and $\alpha_1\beta_3$ receptors using a GABA EC_{3-5} concentration. (B) Typical traces for modulation of chloride currents through $\alpha_1\beta_1$, $\alpha_1\beta_2$ and $\alpha_1\beta_3$ channels by 30 µM VA-A at GABA EC_{3-5}. Control currents (GABA, single bar) and corresponding currents elicited by co-application of GABA and the indicated VA-A concentration (double bar) are shown. GABA, γ-aminobutyric acid; I_{GABA}, GABA-induced chloride currents; VA-A, valerenic acid amide.

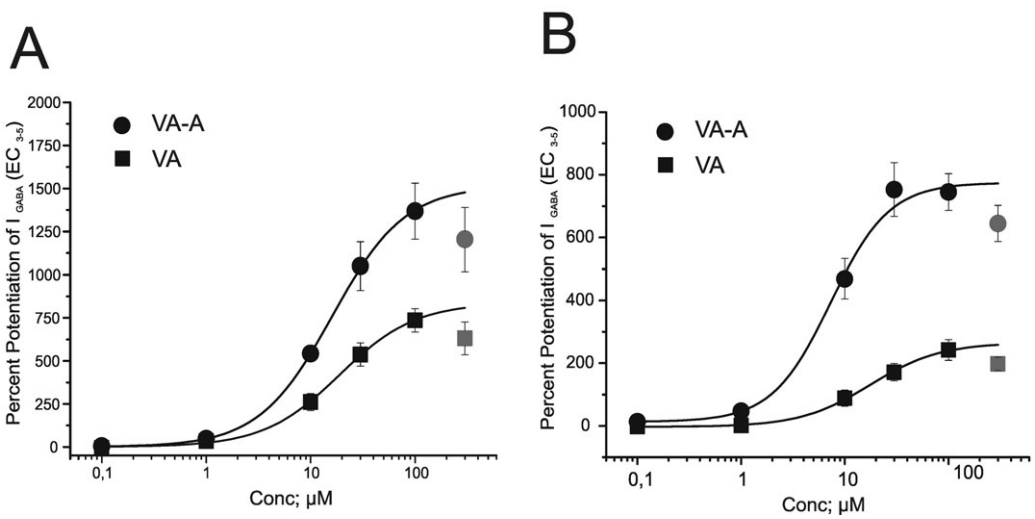


Figure 4

Concentration–effect curves for the enhancement of I_{GABA} through $GABA_A$ receptors composed of (A) $\alpha_1\beta_2\gamma_{2S}$ subunits and (B) of $\alpha_3\beta_3\gamma_{2S}$ subunits by VA-A and VA using a GABA EC_{3-5} (EC_{50} and maximal stimulation are given in Table 2). I_{GABA} at 300 µM (grey symbols) were excluded from the fit. GABA, γ-aminobutyric acid; $GABA_A$, GABA type A; I_{GABA}, GABA-induced chloride currents; VA, valerenic acid; VA-A, valerenic acid amide.

of the total time on the open arms, than the control littermates (injected with solvent), indicating anxiolytic potential of VA (Figure 5). More marked anxiolytic effects were observed after VA-A treatment ($P < 0.01$ vs. control and $P < 0.05$ vs. VA). Treatment with VA-EA, VA-DEA and VA-EE induced anxiolytic effects comparable to VA, while VA-MO did not show any anxiolytic response at the tested dose (Figure 5).

Dose-dependent reduction of anxiety in mice by VA and VA-A

Valerenic acid and VA-A were studied in more detail. As shown in Figure 6A,B, both compounds displayed similar anxiolytic activity at a dose of 1 mg·kg^{-1}. However, the anxiolytic effect was significantly more pronounced for VA-A at 3 mg·kg^{-1}. At higher doses, a reduction of the time spent in the open arms was observed for both compounds.

Table 2

Summary of potencies (as EC_{50}) and efficacies (as maximum stimulation of I_{GABA}) of VA and VA-A on different $GABA_A$ receptor subtypes

Subunit composition	Compound	EC_{50} (μM)	Maximum stimulation of I_{GABA} (%)	Number of experiments (*n*)
$\alpha_1\beta_2\gamma_{2S}$	VA-A	16.0 ± 4.4	1517 ± 234	5
$\alpha_1\beta_2\gamma_{2S}$	VA	18.9 ± 8.5	838 ± 157	4
$\alpha_3\beta_3\gamma_{2S}$	VA-A	7.3 ± 1.9	775 ± 62	6
$\alpha_3\beta_3\gamma_{2S}$	VA	17.6 ± 9.8	265 ± 68	4

GABA, γ-aminobutyric acid; $GABA_A$, GABA type A; I_{GABA}, GABA-induced chloride currents; VA, valerenic acid; VA-A, valerenic acid amide.

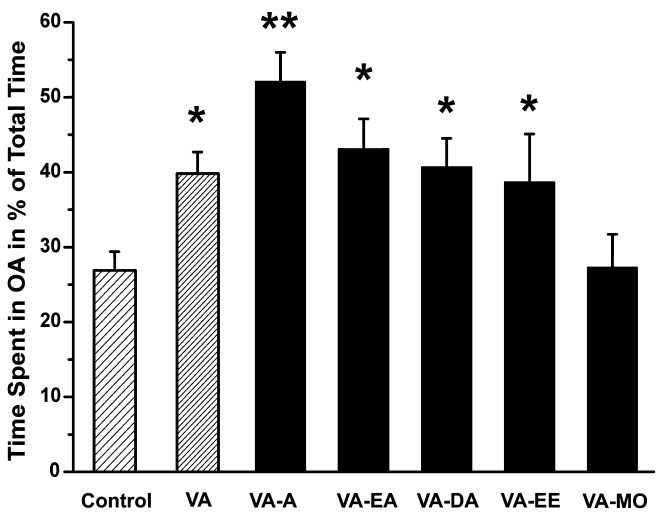


Figure 5

Behaviour in the elevated plus maze test for control and drug-treated mice at a dose of 3 mg·kg^{-1} of the indicated VA derivative. Bars indicate the time spent on the open arms (OA) in % of the total time. Bars represent means ± SEM from at least eight different mice. *P < 0.05; **P < 0.01, significantly different from control. VA, valerenic acid; VA-A, valerenic acid amide; VA-DEA, valerenic acid diethylamide; VA-EA, valerenic acid ethylamide; VA-EE, valerenic acid ethyl ester; VA-MO, valerenic acid morpholine amide.

Reduced exploratory drive after the highest dose of VA-A (30 mg·kg^{-1}) was accompanied by a reduction of closed arm entries and total distance. The number of closed arm entries and total distance of VA-treated mice were not significantly different from control values, at doses between 1 and 10 mg·kg^{-1}. At the highest dose (30 mg·kg^{-1} VA), the number of closed arm entries was reduced (see Figure 6C–F).

Discussion

Synthesis of high efficacy VA derivatives

We and others have recently identified VA (a major constituent of *V. officinalis* L.) as an allosteric modulator of $GABA_A$ receptors selectively enhancing I_{GABA} through channels comprising β_2 or β_3 (but not β_1) subunits (Khom *et al.*, 2007; Benke *et al.*, 2009). In the present study, we have analysed the structure–activity relationship of VA by modifying its carboxyl group (Figure 1). This approach allowed us to generate VA derivatives modulating $GABA_A$ receptors with higher efficacy than the natural parent compound. Significantly higher efficacies in the stimulation of I_{GABA} through $\alpha_1\beta_3$ receptors were observed for VA-A (2247 ± 252%), VA-MA (2298 ± 312%) and VA-EA (1678 ± 258%). All values were significantly different from those for VA ($P < 0.05$). VA-A was even more potent than VA ($P < 0.05$; Table 1). Introduction of bulkier residues or cyclic residues resulted in either a less efficient potentiation of I_{GABA} compared with VA or even a significantly reduced potency (for details, see Table 1 and Figure 2).

With respect to structure–activity relationships we observed no correlation between potency and efficacy of the compounds. The unsubstituted amide VA-A displayed the lowest EC_{50} (highest potency) at the $GABA_A$ receptors investigated. Notably, all mono- and dialkyl-substituted amides and VA itself exhibited almost identical EC_{50} values. This indicates, that, for this part of the molecule, neither charge nor H-bond donor properties nor lipophilicity influence drug receptor binding. In an activity ranking, this cluster of equally active compounds is followed by the ethyl ester, which was slightly less active than the respective ethyl amide. Interestingly, both the piperidine and the morpholine analogues wee significantly less potent than all the other compounds. This might either indicate a steric hindrance caused by the rigid six membered rings or an unfavourable entropic contribution caused by the greater rigidity of the amide substituent.

With respect to maximum stimulation, the differences are more pronounced with almost one order of magnitude difference (ester vs. unsubstituted amide). In addition, a different structure–activity relationship was observed. Open chain

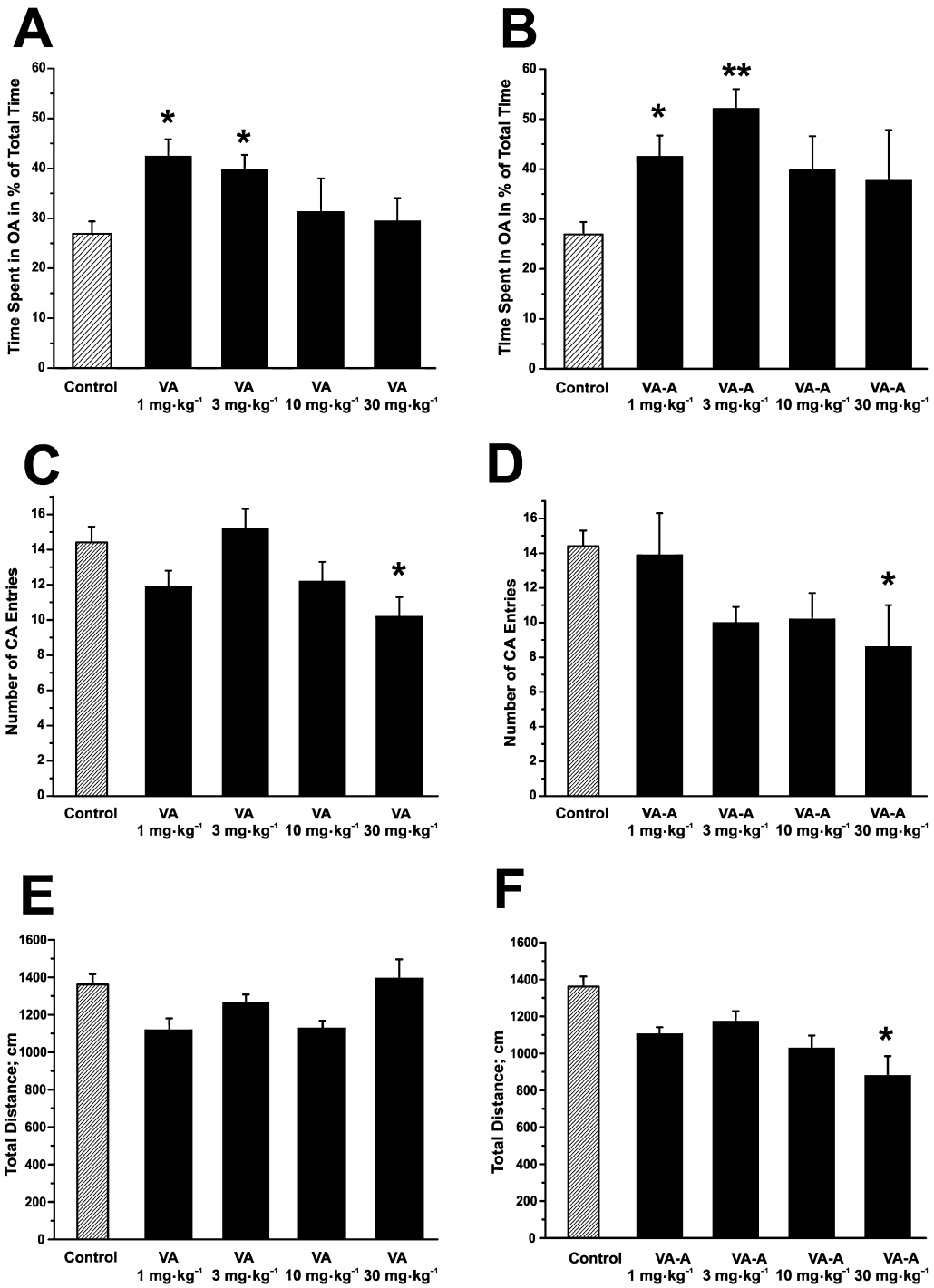


Figure 6

Behaviour in the elevated plus maze test for control (shaded bars in A–F) and drug-treated mice at the indicated dose and compound. Bars indicate in (A) time spent in open arms (OA), in % of the total time after application of the indicated dose of VA, (B) time spent in open arms in % of the total time after application of the indicated dose of VA-A, (C) number of closed arm (CA) entries after application of the indicated dose of VA, (D) number of closed arm entries after application of the indicated dose of VA-A, (E) total distance after application of the indicated dose of VA and (F) total distance after application of the indicated dose of VA-A. Bars represent means ± SEM from at least eight different mice. *$P < 0.05$; **$P < 0.01$, significantly different from control. VA, valerenic acid; VA-A, valerenic acid amide.

secondary and tertiary amides show a clear inverse correlation between logP values and maximum stimulation ($r^2 = 0.83$, $n = 7$), with the unsubstituted amide and the methylamide being the most active compounds. Pairwise comparison of mono- and di-substituted amides also reveals that the latter generally show lower maximum stimulation. This might point towards a potential importance of H-bond donor features in this region. Furthermore, the ethyl ester is almost one order of magnitude less efficient than the analogous ethyl amide, which might indicate that the carbonyl group functions as an H-bond acceptor. However, this data set needs to be interpreted very cautiously, as it is too limited to derive any quantitative (QSAR) models.

Derivatization preserves subunit specificity

Derivatization might influence the pharmacological properties of the compounds, which could lead to a loss of subunit specificity. We have therefore investigated the effects of VA-A (the most potent and effective derivative) on $\alpha_1\beta_1$, $\alpha_1\beta_2$ and $\alpha_1\beta_3$ receptors. Compared with VA, the selectivity for VA-A for $GABA_A$ receptors comprising β_2 or β_3 subunits was conserved. As shown in Figure 3, VA-A induced pronounced modulation of $\alpha_1\beta_3$ and $\alpha_1\beta_2$ receptors, and no significant effect was observed for $\alpha_1\beta_1$ receptors (300 μM; $P < 0.05$). Benke *et al*. (2009) reported, that $\alpha_1\beta_2$ receptors were not modulated by VA. This is in contrast to our previous data (Khom *et al*., 2007) and was also not observed for VA-A in the present study (see Figure 3).

VA-A efficiently enhances I_{GABA} through $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_3\beta_3\gamma_{2S}$ receptors

In line with the data obtained on $\alpha_1\beta_3$ receptors (Figure 2), we found that I_{GABA} through $\alpha_1\beta_2\gamma_{2S}$ receptors were more effectively enhanced by VA-A than by VA (Figure 4A, Table 2). However, our data also show that VA-A was more potent on $GABA_A$ receptors comprising α_3 subunits than α_1 subunits ($P < 0.05$, see Table 2, Figure 4A,B) that could, by analogy with the effects of the benzodiazepines (Rudolph *et al*., 1999; McKernan *et al*., 2000; Atack, 2005; Dias *et al*., 2005), contribute to the anxiolytic action of VA-A (Figure 6).

VA-A showed the most marked anxiolytic effects in mice

Effects on anxiety were investigated in the elevated plus maze for five VA derivatives (VA-A, VA-EA, VA-DEA, VA-EE and VA-MO), selected on the basis of their *in vitro* properties.

In this test, all compounds except VA-MO induced significantly increased ambulation of the open arms. This anxiolytic effect was most pronounced for VA-A, as mice treated with VA-A spent about twice the time in the open arms of the elevated plus maze. Of note is the fact, that increasing lipophilicity did not directly translate into increased behavioural effects. VA-MO was inactive at the dose tested, but effects at higher doses cannot be excluded. Interestingly, VA-EE (the weakest modulator *in vitro*) displayed pronounced anxiolytic responses comparable to that of VA.

In vivo effects of VA and VA-A were compared over a broader range of doses. Both compounds induced comparable anxiolytic effects at 1 $mg \cdot kg^{-1}$. At doses of 10 and 30 $mg \cdot kg^{-1}$, VA treatment failed to increase time spent in the open arms (Figure 6). The lack of increased open arm ambulation after higher doses of VA or VA-A were paralleled by reduced motor activity as indicated by reduced closed arm entries and distance travelled (Figure 6). As this test depends on motor activity, any sedative effects may camouflage anxiolytic effects at higher doses of VA-A.

In conclusion, we report here the synthesis of novel VA derivatives that enabled to gain insights into the structure–activity relationship of this molecule. Amidation was found to increase potency and efficacy of VA. Greater I_{GABA} enhancement by VA-A through receptors comprising β_3 (β_2) subunits *in vitro* correlated with significantly stronger *in vivo* effects as compared with VA. Benke *et al*. (2009) have shown that the anxiolytic activity of VA is absent in β_3(N265M) point-mutated mice. It is therefore likely that the anxiolytic effect of the studied VA derivatives was caused by interaction with $GABA_A$ receptors containing β_3 subunits. Our data thus support the hypothesis that β_3 subunit containing $GABA_A$ receptors are interesting targets for the development of anxiolytics. We can not exclude, however, that the higher potency of VA-A on $GABA_A$ receptors comprising α_3 subunits (Figure 4B) contributes to its anxiolytic action. Some $\beta_{2/3}$-selective $GABA_A$ receptor ligands have also been shown to induce anticonvulsant (Wingrove *et al*., 1994; Groves *et al*., 2006) and anaesthetic effects (Li *et al*., 2006; Drexler *et al*., 2009). It will be interesting to study if such effects can be detected for VA or its derivatives.

Acknowledgements

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Conflicts of interest

The University of Vienna has filed a European Patent Application, serial number EP09151278, with four inventors (S.K., J.R., J.M. and S.H.).

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