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DESIGN, SYNTHESIS AND BIOLOGICAL PROFILE OF MIXED OPIOID AGONIST/N-VGCC BLOCKER PEPTIDES

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METHODS AND MATERIALS

Chemistry

All solvents and coupling reagents were purchased from VWR (Radnor, PN, USA). Fmoc amino acids and the CTC-resin (0.60 mmol/g) were purchased from Chem-Impex (Wood Dale, IL, USA). For orthogonal protection we used OBn for side chain of tyrosine. The intermediate peptide was synthesized by Fmoc-SPPS (standard solid phase peptide synthesis) using TBTU/HOBt for coupling reactions and piperidine 20% solution in DMF for Fmoc group deprotection as previously described.[18] The final products were obtained in solution starting from the intermediate peptide, which was firstly converted in its *tert*-butylamide at the *C*-terminal, the Boc group removed in acidic conditions and then submitted to the following peptide elongation. The purity was found to be ≥ 95% by RP-HPLC. Nuclear magnetic resonance (NMR) spectra of the final compounds were recorded on a Varian Inova 300MHz spectrometer using DMSO-d6 as solvent. The mass spectrometry (MS) system used consisted of an LCQ (Thermo Finnigan) ion trap mass spectrometer (San Jose, CA) equipped with an electrospray ionization (ESI) source. The capillary temperature was set at 300 °C and the spray voltage at 4.25 kV. The fluid was nebulized using nitrogen (N_2) as both the sheath gas and the auxiliary gas. Purification of compounds was carried out by RP-HPLC using a Waters XBridge Prep BEH130 C18, 5.0 μm, 250 mm × 10 mm column at a flow rate of 4.0 mL/min on a Waters Binary pump 1525, and a linear gradient of H_2O/acetonitrile 0.1% TFA ranging from 5% acetonitrile to 90% acetonitrile for 45 min.

In vitro Biological evaluation

Chemicals

Tris-HCl, EGTA, NaCl, $MgCl_2$, GDP, the GTP analogue GTPγS were purchased from Sigma-Aldrich (Budapest, Hungary). The highly selective MOR agonist enkephalin analog

Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) and nociceptin were obtained from Bachem Holding AG (Bubendorf, Switzerland), the highly selective DOR agonist Ile^{5,6}-deltorphin II (IleDelt II) was synthesized in the Laboratory of Chemical Biology group of the Biological Research Center (BRC, Szeged, Hungary). Test ligands were dissolved in DMSO (DAMGO and IleDelt II in water) and were stored in 1 mM stock solution at -20˚C. The radiolabeled GTP analogue, [^{35}S]GTPγS (specific activity: 1000 Ci/mmol) was purchased from Hartmann Analytic (Braunschweig, Germany). [^{3}H]DAMGO (specific activity: 38.8 Ci/mmol), [^{3}H]IleDelt II (specific activity: 19,6 Ci/mmol),[19] were radiolabeled by the Laboratory of Chemical Biology group in BRC (Szeged, Hungary).

Animals

For membrane homogenate preparations male Wistar rats (250-300 g body weight) were used. Rats were housed in the local animal house of BRC (Szeged, Hungary). Animals were kept in a temperature controlled room (21-24^{o}C) under a 12:12 light and dark cycle and were provided with water and food *ad libitum*. All housing and experiments were conducted in accordance with the European Communities Council Directives (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§). The total number of animals as well as their suffering was minimized.

Rat membrane preparations

Rats were decapitated and their brains quickly removed. The brains were prepared for membrane preparation according to Benyhe *et al.*[20] and used for competition binding experiments or partly were further prepared for the [^{35}S]GTPγS binding experiments based on Zádor *et al*.[21]

Briefly, the brains were homogenized, centrifuged in ice-cold 50 mM Tris-HCl (pH 7.4) buffer and incubated at 37^{o}C for 30 min in a shaking water-bath. After incubation the centrifugation was repeated and the final pellet suspended in 50 mM Tris-HCl pH 7.4 buffer containing 0.32 M sucrose and stored at -80^{o}C. For the [^{35}S]GTPγS binding experiments the

final pellet of rat brain membrane homogenate was suspended in ice-cold TEM (Tris-HCl, EGTA, $MgCl_2$) and stored at -80°C for further use.

Radioligand competition binding experiments

Initially the test compounds were incubated together with rat brain membranes containing 0.3-0.5 mg/mL of protein in 3 and 10 μM concentrations, and with ~1 nM [^{3}H]DAMGO or [^{3}H]IleDelt II in 35˚C for 45 min. This was for screening purposes, and if the specific binding of either radioligand was inhibited to the non-specific binding level (0%), the test compounds were further investigated in concentration dependency (0.1 nM-10 μM) with the same conditions for radioligand specific binding inhibition to determine the affinity values ($logIC_{50}$, K_i). Additionally, unlabelled DAMGO and IleDelt II were also incubated together with their labelled counterparts in 3 and 10 μM concentrations for control. Total and non-specific binding was determined in the absence of ligands and presence of 10 μM unlabeled naloxone, respectively. Following the incubation, the bound and unbound radioligands were separated by rapid vacuum filtration through Whatman GF/C glass fibers and washed three times with 5 mL ice cold 50 mM Tris-HCl (pH 7.4). The radioactivity of the filters was detected in UltimaGold™ MV aqueous scintillation cocktail with Packard Tricarb 2300TR liquid scintillation counter. The competition binding assays were performed in duplicate and repeated at least three times.

Functional [^{35}S]GTPγS binding experiments

In [^{35}S]GTPγS binding experiments we measure the GDP→GTP exchange of the $G_{\alpha i/o}$ protein in the presence of a given ligand to measure ligand potency and the maximal efficacy of receptors G-protein. The nucleotide exchange is monitored by a radioactive, non-hydrolysable GTP analogue, [^{35}S]GTPγS. The functional [^{35}S]GTPγS binding experiments were performed as previously described,[22] with modifications. The test compounds at 10 μM were incubated together with rat brain membrane homogenates in the absence or presence of 10 μM DAMGO or IleDelt II containing ~10 μg/mL protein at 30 ˚C for 60 min in Tris-

EGTA buffer (pH 7.4) composed of 50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, containing 20 MBq/0.05 cm^3 [^{35}S]GTPγS (0.05 nM). DAMGO and IleDelt II alone were also measured at 10 μM for control. Total binding was measured in the absence of test compounds; non-specific binding was determined in the presence of 10 □M unlabelled GTPγS. The bound and unbound [^{35}S]GTPγS was separated as described in the competition binding assays section through Whatmann GF/B glass fibers. The radioactivity of the filters was also detected as described above. [^{35}S]GTPγS binding experiments were performed in triplicates and repeated at least three times.

Data analysis

The specific binding of all radiolabelled compounds was calculated by the subtraction of non-specific binding from total binding and was given in percentage. The data was normalized to total specific binding, which was settled 100%, which in case of [^{35}S]GTPγS also represents the basal activity of the G-protein. Data obtained from competition binding experiments where the compounds fully displaced the appropriate radioligand were fitted with the professional curve fitting program, GraphPad Prism 5.0 (GraphPad Prism Software Inc., San Diego, CA), using non-linear regression, 'One-site competition' equation to determine the logIC_{50} value (unlabeled ligand affinity). K_i value was calculated using the Cheng-Prusoff equation.[23] Statistical analyses were performed with GraphPad Prism 5.0 applying one-way ANOVA with Tukey's multiple comparison test. Significance was accepted at the $P < 0.05$ level.

Calcium influx assay

Ca^{2+} responses were measured using FLIPR^{TETRA} and Calcium 4 dye (Molecular Devices). SH-SY5Y cells were plated at 40,000 cells per well in 384 well flat clear-bottom black plate (Corning, NY, USA) and cultured at 37°C in a humidified 5% CO_2 incubator 48 h before assay. The medium was removed and cells loaded with 20 µl/well Calcium 4 dye reconstituted in assay buffer containing (in mM) 140 NaCl, 11.5 glucose, 5.9 KCl, 1.4 $MgCl_2$, 1.2 NaH_2PO_4, 5 $NaHCO_3$, 1.8 $CaCl_2$ and 10 HEPES pH 7.4 and incubated for 30 min at 37°C in a humidified 5% CO_2 incubator. For a specific N-type response in SHSY5Y cells, 10 µM nifedipine (a Ca_V1 blocker) was added to the dye. Ca^{2+} fluorescence responses were recorded at excitation 470-495 nm and emission 515-575 nm for 10 s to set the baseline, 600 s after addition of compound, and for a further 300 s after addition of 90 mM KCl for $Ca_V2.2$ and 40 mM KCl for $Ca_V3.2$. Control for inhibition used CVID for $Ca_V2.2$. Curve fitting was achieved using GraphPad Prism Version 6 (GraphPad Software Inc, San Diego, CA, USA) using nonlinear regression with log[inhibitor] versus normalized response and variable Hill slope for dose-responses. Data were represented as mean ± SEM.

In vivo assays

Animals

Male CD-1 mice (Harlan, Italy) weighing 25-30 g were used for all experiments. Mice were housed for at least 1 week before experimental sessions in colony cages (7 mice in each cage) under standard light (light on from 7.00 a.m. to 7.00 p.m.), temperature (21 ± 1°C), relative humidity (60±10%) with food and water available *ad libitum*. The research protocol was approved by the Service for Biotechnology and Animal Welfare of the Istituto Superiore di Sanità and authorized by the Italian Ministry of Health, according to Legislative Decree 116/92, which implemented the European Directive 86/609/EEC on laboratory animal protection in Italy. Animal welfare was routinely checked by veterinarians

from the Service for Biotechnology and Animal Welfare. The authors further ensure that all efforts were made to minimize animal suffering and the number used.

Intrathecal administration

For intrathecal (i.t.) drug delivery, the mice were briefly anesthetized with halothane (2.5%) and a 30-gauge needle attached to a 25 μL microsyringe was inserted between L5-L6 vertebrae. A sudden advancement of the needle accompanied by a slight flick of the tail was used as the indicator for the proper insertion into the subarachnoid space. Peptides or its vehicle in 5-μL volume was then injected slowly.

Formalin test

The procedure applied has been previously described.[22,24] Briefly, s.c. injection of a dilute solution of formalin (1%, 20 μL/paw) into the mice hind paw evokes nociceptive behavior: licking, biting the injected paw or both, which are considered indices of pain. The nociceptive response shows a biphasic trend, consisting of an early phase from 0 to 10 min after the formalin injection, due to the direct stimulation of peripheral nociceptors, followed by a late prolonged phase occurring from 10 to 40 min, that reflects the response to inflammatory pain. During the test, the mouse was placed in a Plexiglas observation cage (30×14×12 cm), 1 h before the formalin administration to allow it to acclimatize to its surroundings. The total time (s) that the animal spent licking or biting its paw during the formalin-induced early and late phase of nociception was recorded. Experiments were performed 10 min after i.t. treatment.

Tail flick test

The tail flick latency was obtained using a commercial apparatus (Ugo Basile, Italy), equipped by an infrared radiant light source (100 W, 15 V bulb) focused to a photocell utilizing an aluminum parabolic mirror. During the trials the mice were gently hand-restrained with a glove. Radiant heat was focused 3-4 cm from the tip of the tail, and the latency (s) of the tail flick recorded. The measurement was interrupted if the latency

exceeded the cut off time (15 s at 15 V). Baseline latency was calculated as mean of three readings recorded before testing at intervals of 15 min and the time course of latency determined at 15, 30, 45, 60, 90 and 120 min after i.t. treatment. Data obtained in the experiments, were expressed as percentage of maximum effect (% MPE) = (post drug latency-baseline latency)/(cut-off time-baseline latency) x 100.

Data analysis and statistics

Experimental data were expressed as mean ± S.E.M. The significance among groups was evaluated with the analysis of variance followed by Dunnett's correction for multiple comparisons using the statistical software GraphPad Prism 6.03. Statistical significance was assumed at $P < 0.05$.

Characterization of compounds 5-9.

TFA·NH_2Phe-*N*(Me)Leu-Tyr(OBz)-NH*tert*Bu (**4**): 60% yield; ^{1}H-NMR (DMSO-*d6*) δ: 0.707-0.865 (m, $^{\beta}CH_2$ Leu + $^{\gamma}$CH Leu), 1.172 (s, 3CH_3 *tert*-butyl), 1.207 (d, 2CH_3 Leu), 1.451 (m, $^{\beta}CH_2$ Leu), 2.516 (s, *N*CH_3 Leu), 2.709-2.940 (m, $^{\beta}CH_2$Tyr + $^{\beta}CH_2$Phe), 4.387 (m, $^{\alpha}$CH Leu), 4.869 (m, $^{\alpha}$CH Phe + $^{\alpha}$CH Tyr), 4.998 (s, CH_2 benzyl), 7.039-7.441 (m,14H Tyr, Phe aromatics), 8.189 (d, $^{\alpha}$N*H* Tyr), 8.295 (d, $^{\alpha}N{H_2}$ Phe), ; ESI-LRMS $C_{38}H_{48}N_4O_4$ [M] = 600.3; m/z: 601.81 $[M+H]^+$.

TFA·NH_2-Tyr-D-Ala-Gly-Phe-*N*(Me)Leu-Tyr(OBz)-NH*tert*Bu (**5a**): 24% yield; ^{1}H-NMR (DMSO-*d6*) δ: 0.707-0.865 (m, $^{\beta}CH_2$ Leu + $^{\gamma}$CH Leu), 1.172 (s, 3CH_3 *tert*-butyl), 1.19 (d, CH_3, D-Ala), 1.207 (d, 2CH_3 Leu), 1.451 (m, $^{\beta}CH_2$ Leu), 2.516 (s, *N*CH_3 Leu), 2.779-2.331 (m, $^{\beta}CH_2$Tyr + $^{\beta}CH_2$Phe), 3.953 (m, $^{\alpha}$CH, D-Ala), 4.311 (m, $^{\alpha}$CH, Tyr), 4.479 (d, $^{\alpha}$CH, Gly), 4.387 (m, $^{\alpha}$CH Leu), 4.869 (m, $^{\alpha}$CH Phe + $^{\alpha}$CH Tyr), 4.998 (s, CH_2 benzyl), 6.693-7.321 (m, 18 H Tyr, Phe aromatics), 7.521 (t, $^{\alpha}$N*H* Gly), 7.73 (d, $^{\alpha}$N*H* D-Ala), 8.055 (d, $^{\alpha}NH_2$ Tyr), 8.189 (d, $^{\alpha}$N*H* Tyr), 8.211 (d, $^{\alpha}$N*H* Phe), 9.348 (s, OH Tyr); ESI-LRMS $C_{50}H_{65}N_7O_8$ [M] = 891.4; m/z: 892.5 $[M+H]^+$.

TFA·NH_2-Tyr-D-Ala-Gly-Phe-*N*(Me)Leu-Tyr(OBz)-NH*tert*Bu (**5b**): 29% yield; ^{1}H-NMR (DMSO-*d6*) δ: 0.724-0.795 (m, $^{\beta}CH_2$ Leu + $^{\gamma}$CH Leu), 1.172 (s, 3CH_3 *tert*-butyl), 1.19 (d, CH_3, D-Ala), 1.207 (d, 2CH_3 Leu), 1.648 (m, $^{\beta}CH_2$ Leu), 2.516 (s, *N*CH_3 Leu), 2.231-2.375 (m, $^{\beta}CH_2$Tyr + $^{\beta}CH_2$Phe), 3.578 (m, $^{\alpha}$CH, D-Ala), 4.913 (m, $^{\alpha}$CH, Tyr), 4.399 (d, $^{\alpha}$CH, Gly), 4.112 (m, $^{\alpha}$CH Leu), 4.944 (m, $^{\alpha}$CH Phe + $^{\alpha}$CH Tyr), 4.998 (s, CH_2 benzyl), 6.693-7.321 (m, 18 H Tyr, Phe aromatics), 7.421 (t, $^{\alpha}$N*H* Gly), 7.73 (d, $^{\alpha}$N*H* D-Ala), 8.055 (d, $^{\alpha}NH_2$ Tyr), 8.189 (d, $^{\alpha}$N*H* Tyr), 8.211 (d, $^{\alpha}$N*H* Phe), 9.348 (s, OH Tyr); ESI-LRMS $C_{50}H_{65}N_7O_8$ [M] = 891.4; m/z: 892.5 $[M+H]^+$.

TFA·NH_2-Tyr-D-Ala-Gly-Phe-Phe-*N*(Me)Leu-Tyr(OBz)-NH*tert*Bu (**6a**): 40% yield; ^{1}H-NMR (DMSO-*d6*) δ: 0.707-0.865 (m, $^{\beta}CH_2$ Leu + $^{\gamma}$CH Leu), 1.172 (s, 3CH_3 *tert*-butyl), 1.19 (d, CH_3, D-Ala), 1.207 (d, 2CH_3 Leu), 1.451 (m, $^{\beta}CH_2$ Leu), 2.516 (s, *N*CH_3 Leu), 2.612-2.436 (m, $^{\beta}CH_2$Tyr + $^{\beta}CH_2$Phe), 3.953 (m, $^{\alpha}$CH, D-Ala), 4.311 (m, $^{\alpha}$CH, Tyr), 4.479 (d, $^{\alpha}$CH, Gly), 4.387 (m, $^{\alpha}$CH Leu),

4.7113-4.8051 (m, $^{\alpha}$CH Phe + $^{\alpha}$CH Tyr), 4.998 (s, CH_2 benzyl), 6.693-7.811 (m, 23 H Tyr, Phe aromatics), 7.521 (t, $^{\alpha}$N*H* Gly), 7.73 (d, $^{\alpha}$N*H* D-Ala), 8.071 (d, $^{\alpha}NH_2$ Tyr), 8.158 (d, 2$^{\alpha}$N*H* Phe), 8.621 (d, $^{\alpha}$N*H* Tyr), 9.348 (s, OH Tyr); ESI-LRMS $C_{59}H_{74}N_8O_9$ [M] = 1038.5; m/z: 1039.7 $[M+H]^+$.

TFA·NH_2-Tyr-D-Ala-Gly-Phe-Phe-*N*(Me)Leu-Tyr(OBz)-NH*tert*Bu (**6b**): 32% yield; ^{1}H-NMR (DMSO-*d6*) δ: 0.711-0.863 (m, $^{\beta}CH_2$ Leu + $^{\gamma}$CH Leu), 1.172 (s, $3CH_3$ *tert*-butyl), 1.19 (d, CH_3, D-Ala), 1.207 (d, $2CH_3$ Leu), 1.501 (m, $^{\beta}CH_2$ Leu), 2.516 (s, *N*CH_3 Leu), 2.713-2.866 (m, $^{\beta}CH_2$Tyr + $^{\beta}CH_2$Phe), 3.988 (m, $^{\alpha}$CH, D-Ala), 4.311 (m, $^{\alpha}$CH, Tyr), 4.576 (d, $^{\alpha}$CH, Gly), 4.817 (m, $^{\alpha}$CH Leu), 4.889-4.951 (m, $^{\alpha}$CH Phe + $^{\alpha}$CH Tyr), 5.118 (s, CH_2 benzyl), 6.693-7.811 (m, 23 H Tyr, Phe aromatics), 7.621 (t, $^{\alpha}$N*H* Gly), 7.53 (d, $^{\alpha}$N*H* D-Ala), 8.071 (d, $^{\alpha}NH_2$ Tyr), 8.158 (d, 2$^{\alpha}$N*H* Phe), 8.621 (d, $^{\alpha}$N*H* Tyr), 9.348 (s, OH Tyr); ESI-LRMS $C_{59}H_{74}N_8O_9$ [M] = 1038.5; m/z: 1039.7 $[M+H]^+$.

TFA·NH_2-Tyr-D-Ala-Gly-Phe-β-Ala-Phe-*N*(Me)Leu-Tyr(OBz)-NH*tert*Bu (**7**): 7% yield; ^{1}H-NMR (DMSO-*d6*) δ: 0.707-0.865 (m, $^{\beta}CH_2$ Leu + $^{\gamma}$CH Leu), 1.172 (s, $3CH_3$ *tert*-butyl), 1.19 (d, CH_3, D-Ala), 1.207-1.409 (d, $2CH_3$ Leu, m, βCH_2 Ala), 1.451 (m, $^{\beta}CH_2$ Leu), 2.516 (s, *N*CH_3 Leu), 2.612-2.436 (m, $^{\beta}CH_2$Tyr + $^{\beta}CH_2$Phe), 3.812-3.953 (m, $^{\alpha}$CH, D-Ala, β-Ala), 4.311 (m, $^{\alpha}$CH, Tyr), 4.479 (d, $^{\alpha}$CH, Gly), 4.387 (m, $^{\alpha}$CH Leu), 4.711-4.805 (m, $^{\alpha}$CH Phe + $^{\alpha}$CH Tyr), 4.998 (s, CH_2 benzyl), 6.693-7.811 (m, 23 H Tyr, Phe aromatics), 7.521 (t, $^{\alpha}$N*H* Gly), 7.73 (d, $^{\alpha}$N*H* D-Ala), 7.803 (t, N*H* β-Ala), 8.071 (d, $^{\alpha}NH_2$ Tyr), 8.158 (d, 2$^{\alpha}$N*H* Phe), 8.621 (d, $^{\alpha}$N*H* Tyr), 9.348 (s, OH Tyr); ESI-LRMS $C_{62}H_{79}N_9O_{10}$ [M] = 1109.5; m/z: 1110.1 $[M+H]^+$.

TFA·NH_2-Tyr-D-Ala-Gly-Phe-Gaba-Phe-*N*(Me)Leu-Tyr(OBz)-NH*tert*Bu (**8**): 25% yield; ^{1}H-NMR (DMSO-*d6*) δ: 0.707-0.865 (m, $^{\beta}CH_2$ Leu + $^{\gamma}$CH Leu), 1.172 (s, $3CH_3$ *tert*-butyl), 1.19 (d, CH_3, D-Ala), 1.207-1.409 (d, $2CH_3$ Leu, m, βCH_2 Ala), 1.451 (m, $^{\beta}CH_2$ Leu), 1.822 (q, $^{\gamma}$CH2 Gaba), 2.223

(quint, $^{\beta}$CH2 Gaba), 2.516 (s, *N*CH_3 Leu), 2.612-2.436 (m, $^{\beta}CH_2$Tyr + $^{\beta}CH_2$Phe), 3.542 (t, $^{\alpha}$CH2 Gaba), 3.812-3.953 (m, $^{\alpha}$CH, D-Ala, β-Ala), 4.311 (m, $^{\alpha}$CH, Tyr), 4.479 (d, $^{\alpha}$CH, Gly), 4.387 (m, $^{\alpha}$CH Leu), 4.711-4.805 (m, $^{\alpha}$CH Phe + $^{\alpha}$CH Tyr), 4.998 (s, CH_2 benzyl), 6.693-7.811 (m, 23 H Tyr, Phe aromatics), 7.521 (t, $^{\alpha}$N*H* Gly), 7.73 (d, $^{\alpha}$N*H* D-Ala), 7.803 (t, N*H* β-Ala), 7.943 (t, N*H* Gaba), 8.071 (d, $^{\alpha}NH_2$ Tyr), 8.158 (d, 2$^{\alpha}$N*H* Phe), 8.621 (d, $^{\alpha}$N*H* Tyr), 9.348 (s, OH Tyr); ESI-LRMS $C_{63}H_{81}N_9O_{10}$ [M] = 1123.6; m/z: 1124.8 $[M+H]^+$.

TFA·NH_2-Tyr-D-Ala-Gly-Phe-Gly-Phe-*N*(Me)Leu-Tyr(OBz)-NH*tert*Bu (**9**): 6% yield; ^{1}H-NMR (DMSO-*d6*) δ: 0.707-0.865 (m, $^{\beta}CH_2$ Leu + $^{\gamma}$CH Leu), 1.172 (s, 3CH_3 *tert*-butyl), 1.19 (d, CH_3, D-Ala), 1.207 (d, 2CH_3 Leu), 1.451 (m, $^{\beta}CH_2$ Leu), 2.516 (s, *N*CH_3 Leu), 2.779-2.331 (m, $^{\beta}CH_2$Tyr + $^{\beta}CH_2$Phe), 3.953 (m, $^{\alpha}$CH, D-Ala), 4.311 (m, $^{\alpha}$CH, Tyr), 4.411 (d, 2$^{\alpha}$CH, Gly), 4.387 (m, $^{\alpha}$CH Leu), 4.869 (m, $^{\alpha}$CH Phe + $^{\alpha}$CH Tyr), 4.998 (s, CH_2 benzyl), 6.693-7.321 (m, 18 H Tyr, Phe aromatics), 7.521-7.691 (t, 2$^{\alpha}$N*H* Gly), 7.73 (d, $^{\alpha}$N*H* D-Ala), 8.055 (d, $^{\alpha}NH_2$ Tyr), 8.189 (d, $^{\alpha}$N*H* Tyr), 8.211 (d, $^{\alpha}$N*H* Phe), 9.348 (s, OH Tyr); ESI-LRMS $C_{61}H_{77}N_9O_{10}$ [M] = 1195.5; m/z: 1196.7 $[M+H]^+$.

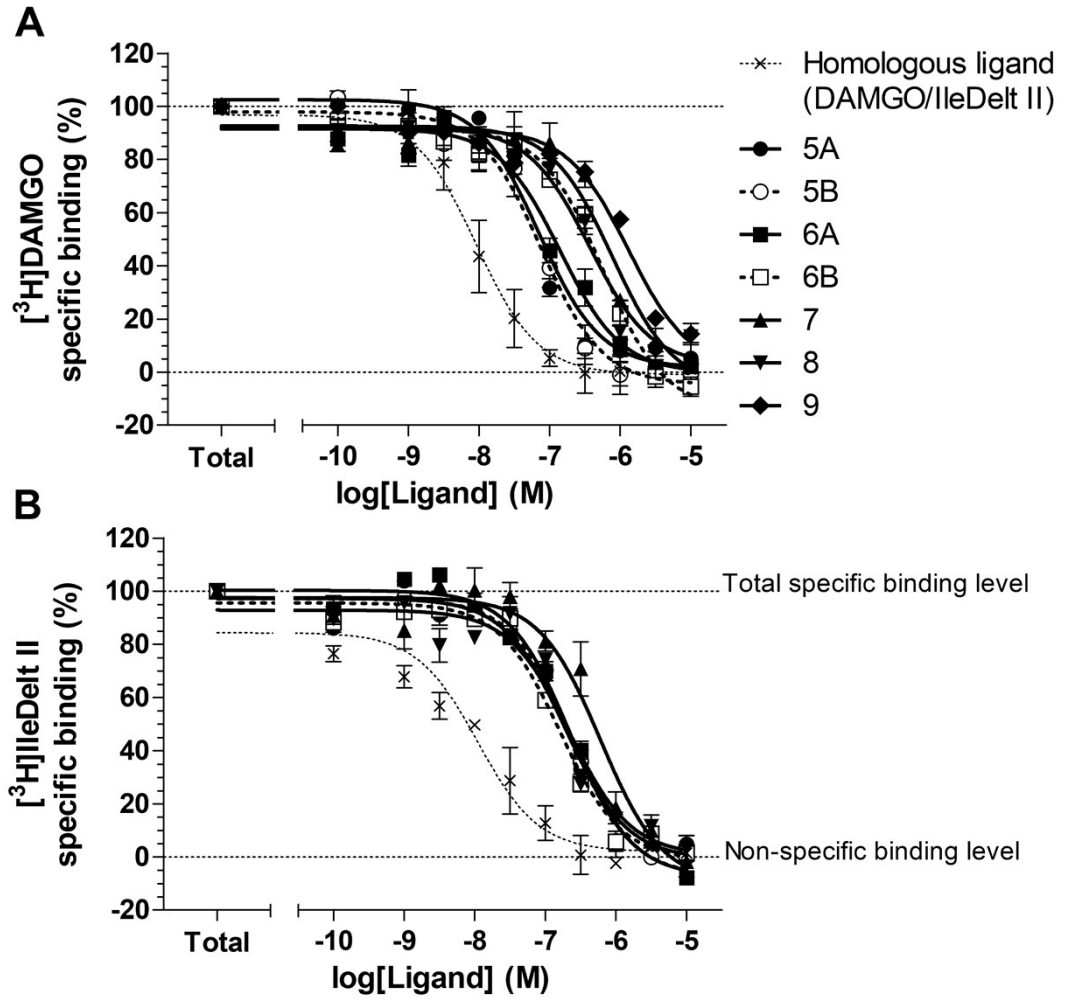

Figure 1S. Competition binding of compounds 5-9 with MOR (A) and DOR (B) radioligands. Figures represents the specific binding of the indicate radioligands in percentage (mean ± S.E.M.) in the presence of increasing concentrations (0.1 nM-10 μM) of the indicated ligands. Total represents the specific binding of the radioligands in the absence of the ligands. The dotted lines indicate the level of total (100%) and non-specific binding (0%). The experiments and data analysis were performed as described under section 'Radioligand competition binding experiments' and 'Data analysis' within the 'Materials and methods'.

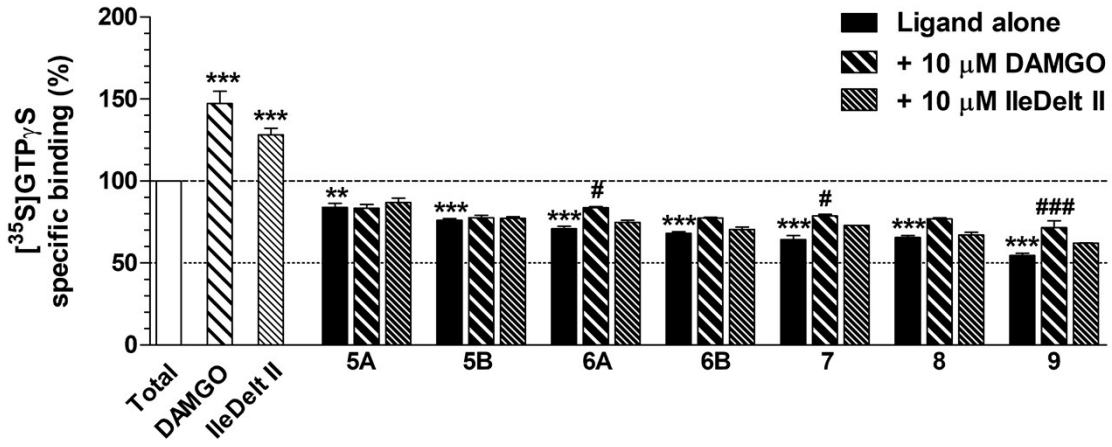

Figure 2S. The G-protein affinity of **5-9** peptides and their MOR and DOR selectivity in [^{35}S]GTPγS binding assays performed in rat brain. Data represents the specific binding of [^{35}S]GTPγS in percentage (mean ± S.E.M.) in the presence of the indicated ligands in 10 μM concentrations in the absence or presence of 10 μM of DAMGO or IleDelt II, which are MOR and DOR selective agonists, respectively. 'Total' represents the specific binding of [^{35}S]GTPγS in the absence of the ligands, which also represents the basal activity of the monitored G-protein. The dotted lines indicate the level of total binding (100%) and 50% [^{35}S]GTPγS specific binding. *: indicates the significant difference compared to total (One-way ANOVA, Tukey multiple comparison post-hoc test). $^{\#}$: indicates the significant difference compared to ligand alone (One-way ANOVA, Tukey multiple comparison post-hoc test). ***/$^{\#\#\#}$: P > 0.001; **: P > 0.01; $^{\#}$: P > 0.05. The experiments and data analysis were performed as described under section 'Functional [^{35}S]GTPγS binding experiments' and 'Data analysis' within the 'Materials and methods'.