Supporting information 1

EC18 AS A TOOL TO UNDERSTAND THE ROLE OF HCN4 CHANNELS IN MEDIATING HYPERPOLARIZATION-ACTIVATED CURRENT IN TISSUES

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1. CHEMISTRY

1.1 General

All melting points were taken on a Büchi apparatus. NMR spectra were recorded on a Bruker Avance 400 spectrometer. In the ^{1}H-NMR spectra the following abbreviations have been used: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet; to label protons or carbon the abbreviations cy (cyclohexane) and bz (benzazepinone) have been also used. Infrared spectra were recorded with a Perkin-Elmer 681 spectrophotometer in Nujol mull for solids and neat for liquids. Thin layer chromatography (TLC) were performed on Kieselgel Merck F254 silica gel plates and on F254 neutral alumina plates. Chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063-0. 200 mm; Merck) or flash cromatography (0.040-0.063 mm; Merck) using the proper eluents. Optical rotation was measured at a concentration of 1g/100mL (c=1) with a Perkin-Elmer polarimeter (accuracy 0.002°). The mass spectra of studied compounds were obtained by using a Varian 1200L triple quadrupole equipped with an electrospray ionization source (ESI), in positive ions monitoring. High resolution mass spectrometry (HR-MS) analysis were performed with a Thermo Finnigan LTQ Orbitrap mass spectrometer equipped with an ESI source. The analysis were carried out introducing, via syringe pump at 10 μL min^{-1}, the sample solution (1.0 μg mL^{-1} in mQ water:acetonitrile 50:50), and it was acquired the signal of the positive ions. These experimental conditions allow the monitoring of protonated molecules of the studied compounds ([M+H]$^{+}$ species), that they were measured with a proper dwell time to achieve 60,000 units of resolution at Full Width at Half Maximum (FWHM). Elemental composition of compounds were calculated on the basis of their measured accurate masses, accepting only results with an attribution error less than 2.5 ppm and a not integer RDB (double bond/ring equivalents) value, in order to consider only the protonated species.[1] The purity of the final compounds was determined by Agilent 1200 liquid chromatography system composed by autosampler, binary pumps, column oven and diode-array detector (LC-DAD) operating in UV range (210-400 nm). The analyses were carried out using a Phenomenex Luna C18 column 50 mm length, 2 mm internal diameter and 3 μm particle size. The analyte separation were ensured employing as mobile phase 10 mM Formic Acid solution (phase A) and 10 mM Formic Acid solution in acetonitrile (phase B) in gradient elution. The time program elution was as follows: initial 10% phase B for 0 min, then increase to 90% phase B in 4 min and kept for 5 min. The analysis performed at constant flow of 0.25 mL min -1, temperature of 40°C and injecting 10 μL of a 10 μg mL^{-1} solution of each analyte. The obtained results displayed that all the studied compound show a purity equal or higher than 95%. The chromatographic profiles of LC-DAD analysis were reported in the Supporting_information_2 file (Figures S19-S25). Compounds were named following IUPAC rules as applied by Marvin Sketch.

3,4-Dimethoxyphenethyl bromide **8a** was commercially available. 3,4-Dimethoxybenzyl chloride **8b**, 4-(2-bromoethoxy)-1,2-dimethoxybenzene **8d** and 4-[(1E)-3-chloroprop-1-en-1-yl]-1,2-dimethoxybenzene **8e** were prepared according to literature procedures.[2-4] 4-(3-Iodopropyl)-1,2-dimethoxybenzene **8c**[5] was prepared from ethyl 3-(3,4-dimethoxyphenyl)propanoate, through $LiAlH_4$ reduction, treatment with thionyl chloride and Cl-I halogen exchange.

1.2 Methyl 5-(7,8-dimethoxy-2-oxo-2,3-dihydro-1H-3-benzazepin-3-yl)cyclohex-3-ene-1-carboxylate 4.

N-bromosuccinimmide (0.034 mol; 6.03 g) and a catalytic amount of AIBN were added to a solution of methyl cyclohex-3-enecarboxylate (0.034 mol; 4.75 g) in CCl_4 (10 mL). The mixture was heated at reflux for 3 hours and monitored by TLC (eluent: cyclohexane-ethyl acetate, 9:1). During the reaction a white precipitate (succinimide) was formed that was filtered off once the reaction was finished. The filtrate was collected and the solvent was removed under vacuum to afford methyl 5-bromocyclohex-3-enecarboxylate[6-7] (7.30 g; yellow oil), which was used in the next reaction without further purification. Potassium *tert*-butoxyde (0.04 mol; 4.48 g) was added under nitrogen to a suspension of 7,8-dimethoxy-1H-benzo-3-azepin-2-one[8] (0.033 mol; 7.23 g) in anhydrous DMSO. The complete formation of the anion was observed because of the formation of a dark red solution. Then, the bromocyclohexene derivative (0.033 mol; 7.3 g), dissolved in DMSO (10 mL) was added dropwise. After stirring overnight at room temperature, DMSO was partially removed under reduced pressure. Ice was then added to the mixture, causing the formation of a brown precipitate (unreacted benzazepinone) which was filtered off. The aqueous layer was extracted with chloroform (3 × 30 mL); the organic layers were collected, dried on Na_2SO_4 and evaporated under vacuum to give a residue that was purified by flash chromatography. Eluting with cyclohexane-ethyl acetate (6:4) afforded 0.47g of *cis*-**4** and 2.31 g of *cis-trans* mixture (23% overall yield of **4**) as oils. *Cis*-**4**: ^{1}H NMR ($CDCl_3$) δ 1.56 (q, J = 12.4 Hz, 1H, H-6_{cy}); 2.13-2.19 (m, 1H, H-6_{cy}); 2.21-2.39 (m, 2H, H-2_{cy}); 2.72-2.82 (m, 1H, H-1_{cy}); 3.39 (d, J = 12.4 Hz, 1H, H-1_{bz}); 3.51 (d, J = 12.4 Hz, 1H, H-1_{bz}); 3.66 (s, 3H, $COOCH_3$); 3.88 (s, 3H, OCH_3); 3.90 (s, 3H, OCH_3); 5.33-5.40 (m, 1H, H-5_{cy}); 5.51 (m, 1H, H-4_{cy}); 5.94-6.01 (m, 1H, H-3_{cy}); 6.21 (d, J = 9.6 Hz, 1H, H-4_{bz}); 6.40 (d, J = 9.6 Hz, 1H, H-5_{bz}); 6.73 (s, 1H, H-6_{bz}); 6.80 (s, 1H, H-9_{bz}) ppm. ^{13}C-NMR ($CDCl_3$, APT) δ 27.23 (C-2_{cy}); 30.29 (C-6_{cy}); 38.85 (C-1_{cy}); 43.40 (C-1_{bz}); 51.34 (C-5_{cy}); 51.78 (COO<u>C</u>H_3); 55.99 (OCH_3); 109.60 (C-6_{bz}); 111.18 (C-9_{bz}); 118.01 (C-5_{bz}); 124.46 (C-4_{bz}); 124.73 (C); 126.47 (C); 127.83 (C-4_{cy}); 129.81 (C-3_{cy}); 148.08 (C); 149.90 (C); 167.56 (C); 174.88 (CO) ppm.

1.3 *cis* Methyl 3-(7,8-dimethoxy-2-oxo-2,3,4,5-tetrahydro-1H-3-benzazepin-3-yl)cyclohexane-1-carboxylate 5

Pd/C (10% p/p, 0.1 g) was added to a solution of *cis-trans*-**4** (8.95 mmol; 3.2 g) in absolute ethanol (50 mL) and hydrogenated in a Parr apparatus at 90 Psi for 3 days at room temperature. The mixture was filtered and the solvent removed under vacuum to give a residue which was purified by flash chromatography. Eluting with dichloromethane-methanol (98:2) afforded ***cis-5*** as a clear oil in 50% yields, together with some *cis-trans* mixture. ^{1}H NMR ($CDCl_3$) δ 1.26-1.38 (m, 1H, H-6_{cy}); 1.38-1.50 (m, 2H, H-4_{cy} + H-5_{cy}); 1.59 (q, J=12.4 Hz, 1H, H-2_{cy}); 1.77 (d, J=9.6 Hz, 1H, H-4_{cy}); 1.89-1.95 (m, 1H, H-5_{cy}); 1.90-2.03 (m, 2H, H-2_{cy}+ H-6_{cy}); 2.51 (t, J = 12.0 Hz, 1H, H-1_{cy}); 3.01-3.08 (m, 2H, H-5_{bz}); 3.68 (s, 3H, $COOCH_3$); 3.72 (t, J=5.6 Hz, 2H, H-4_{bz}); 3.84-3.86 (m, 8H, OCH_3 + H-1_{bz}); 4.55 (t, J = 11.2 Hz, 1H, H-3_{cy}); 6.53 (s, 1H, H-6_{bz}); 6.60 (s, 1H, H-9_{bz}) ppm. ^{13}C-NMR ($CDCl_3$, APT) δ 24.48 (C-5_{cy}); 28.31 (C-6_{cy}); 29.94 (CH-4_{cy}); 32.86 (CH-2_{cy}); 34.03 (C-5_{bz}); 40.41 (C-4_{bz}); 42.52 (C-1_{cy}); 43.09 (C-1_{bz}); 51.28 (C-3_{cy}); 51.69 (COO<u>C</u>H_3); 55.94 (OCH_3); 55.97 (OCH_3); 113.26 (C-6_{bz}); 114.13 (C-9_{bz}); 123.37 (C); 127.16 (C); 147.20 (C); 147.96 (C); 175.47 (CO) ppm.

1.4 *cis* 3-(7,8-dimethoxy-2-oxo-2,3,4,5-tetrahydro-1H-3-benzazepin-3-yl)cyclohexane-1-carboxylic acid 6

A solution of ***cis-5*** (2.12 mmol; 0.79 g) in NaOH 2.5M (2.53 mmol; 1.01 mL) and methanol (15 mL) was stirred for 24 hours at room temperature. The reaction was monitored by TLC (eluent:

dichloromethane-methanol, 9:1). The solvent was evaporated under vacuum and the residue was taken up in water and extracted with ethyl acetate. The water layer was collected, acidified (pH 1) with 2N HCl, and extracted with dichloromethane. Removal of the dried (Na_2SO_4) solvent under vacuum gave **6** as a gummy white solid (0.73 g, quantitative yield). ^{1}H NMR ($CDCl_3$,) δ 1.24-1.48 (m, 3H); 1.57 (q, J=12.4 Hz, 1H); 1.74 (d, J=10.4 Hz, 1H); 1.86-1.93 (m, 1H); 2.01 (d, J=12.0 Hz, 2H); 2.53 (tt, J=12.0 Hz, 3.6 Hz, 1H, H-1_{cy}); 3.01 (m, 2H, H-5_{bz}); 3.72 (t, J = 6.0 Hz, 2H, H-4_{bz}); 3.81 (s, 3H, OCH_3); 3.83 (s, 3H, OCH_3); 3.84 (s, 2H, H-1_{bz}); 4.55 (t, J=12.0 Hz, 1H, H-3_{cy}); 6.52 (s, 1H, H-6_{bz}); 6.60 (s, 1H, H-9_{bz}) ppm.

1.5 *cis* 3-(3-aminocyclohexyl)-7,8-dimethoxy-2,3,4,5-tetrahydro-1H-3-benzazepin-2-one 7

Method A: a solution of **6** (0.1 g, 0.3 mmol), DPPA (0.08 mL, 1.2 eq) and Et_3N (0.05 mL, 1.2 eq) in anhydrous toluene (2 mL) was heated at 110 °C for 3 hrs. The reactions was monitored by IR spectroscopy, following the formation of the isocyanate (ν 2250 cm^{-1}). After removal of the solvent, the residue was treated with THF (10 mL), HCl 2N (3 mL) was added and the mixture was left stirring at room temperature overnight. The solvent was removed under vacuum and the solid residue was washed with ether, then it was dissolved in water, the pH was made alkaline with NaOH 2N and the water solution was extracted twice with CH_2Cl_2. The organic phases were collected, dried (Na_2SO_4) then the solvent was removed leaving amine **7** in 53% yields.

Method B: in a two-necked flask under nitrogen atmosphere, thionyl chloride (13.85 mmol; 1 mL) was added dropwise to a solution of **6** (1.74 mmol; 0.6 g) in chloroform (50 mL). The mixture was heated at 61 °C for 3 hours, then the solvent was removed under vacuum to give a residue that was washed with cyclohexane (2 × 30 mL) and dried under vacuum. Anhydrous acetone (10 mL) was poured into the flask followed by a saturated aqueous solution of NaN_3 (6 mL). After stirring for 10 minutes, a small excess of water was added to the solution. Acetone was evaporated under vacuum and the aqueous layer was extracted with dichloromethane (3 × 15 mL). The organic layers were collected, dried (Na_2SO_4) and evaporated under vacuum to give a gummy solid residue, consisting of *3-(7,8-dimethoxy-2-oxo-2,3,4,5-tetrahydro-1H-3-benzazepin-3-yl)cyclohexane-1-carbonyl azide* (0.54 g; 87%), that was used in the next step without further purification. The formation of acylazide was detected by IR spectroscopy (ν= 2100 cm^{-1}; CON_3).

A solution of this compound (1.29 mmol; 0.48 g) in toluene was heated under reflux to allow the formation of isocyanate. The reaction was monitored by IR spectroscopy and continued until the peak of acylazide (ν 2100 cm^{-1}) disappeared due to the formation of isocyanate (ν 2250 cm^{-1}). The toluene was removed under vacuum to give a residue that was dissolved in THF (3 mL) and 2N HCl (3 mL). The resulting solution was stirred at room temperature overnight. Organic solvents were evaporated under vacuum and the aqueous layer was washed with diethyl ether, basified with 2.5M NaOH and extracted with dichloromethane. The organic layers were collected, dried on Na_2SO_4 and removed under vacuum. **7** was obtained as a gummy solid (0.35 g, 65% yields).

^{1}H NMR ($CDCl_3$) δ 1.01 (dq, J = 12.0 Hz, 3.4 Hz, 1H, H-4_{cy}); 1.22-1.49 (m, 3H, H-2_{cy}+H-6_{cy}+H-5_{cy}); 1.70 (d, J=12.0 Hz, 1H, H-6_{cy}); 1.77-1.92 (m, 5H, NH_2+H-5_{cy}+H-4_{cy}+H-2_{cy}); 2.86 (tt, J=11.0 Hz, J=3.8 Hz, 1H, H-3_{cy}); 3.02 (t, J=6.0 Hz, 2H, H-5_{bz}); 3.69 (t, J=6.0 Hz, 2H, H-4_{bz}); 3.81 (s, 2H, H-1_{bz}); 3.83 (s, 3H, OCH_3); 3.85 (s, 3H, OCH_3); 4.51 (tt, J=12.0 Hz, J=3.6 Hz, 1H, H-3_{cy}); 6.53 (s, 1H, H-6_{bz}); 6.61 (s, 1H, H-9_{bz}) ppm. ^{13}C NMR ($CDCl_3$, APT) δ 23.20 (C-5_{cy}); 29.66 (C-6_{cy}); 33.95 (C-5_{bz}); 35.32 (C-4_{cy}); 40.36 (C-4_{bz}); 40.83 (C-2_{cy}); 43.03 (C-1_{bz}); 49.74 (C-1_{cy}); 50.91 (C-3_{cy}); 55.93

(OCH_3); 113.28 (C-6_{bz}); 114.13 (C-9_{bz}); 123.49 (C); 127.22(C); 147.18 (C); 147.95 (C); 172.05 (CO) ppm.

1.6. Synthesis of compounds 9a-f and 3f

cis **3-(3-{[2-(3,4-dimethoxyphenyl)ethyl]amino}cyclohexyl)-7,8-dimethoxy-2,3,4,5-tetrahydro-1H-3-benzazepin-2-one 9a** A solution of **7** (0.63 mmol; 0.20 g), anhydrous triethylamine (0.94 mmol; 0.13 mL) and 3,4-dimethoxyphenethyl bromide **8a** (0.94 mmol; 0.23 g) in dry DMF (2 mL) was heated at 60 °C overnight under anhydrous conditions. The reaction was monitored by TLC in dichloromethane-methanol (96:4). The mixture was cooled to room temperature and then the solvent was evaporated under vacuum to give a residue that was treated with 2N HCl (3 mL) and washed with diethyl ether (2 × 15 mL). The acidic aqueous layer was basified with a sodium carbonate saturated aqueous solution and extracted with dichloromethane (3 × 15 mL). The organic layers were collected, dried (Na_2SO_4) and evaporated under vacuum to afford a residue that was purified with gravity column chromatography. Eluting with dichloromethane-methanol-ammonia (90:10:0.5) gave **9a** as a clear oil in 49% yields. ^{1}H NMR ($CDCl_3$) δ 1.05 (q, J=11.6 Hz, 1H, H-4_{cy}); 1.22-1.32 (m, 1H, H-2_{cy}); 1.33-1.47 (m, 2H, H-6_{cy}+H-5_{cy}); 1.72 (d, J=10.0 Hz, 1H, H-6_{cy}); 1.83 (d, J = 12.4 Hz, 1H, H-5_{cy}); 1.91 (d, J = 12.4 Hz, 1H, H-4_{cy}); 1.98 (d, J = 11.2 Hz, 1H, H-2_{cy}); 2.65 (t, J=10.9 Hz, 1H, C-3_{cy}); 2.75-2.82 (m, 2H, CH_2N); 2.88-2.96 (m, 2H, CH_2Ar); 2.97-3.06 (m, 2H, H-5_{bz}); 3.67 (t, J=6.0 Hz, 2H, H-4_{bz}); 3.78-3.84 (m, 10H, OCH_3 + H-1_{bz}); 4.50-4.56 (m, 1H, H-1_{cy}); 6.53 (s, 1H, H-6_{bz}); 6.60 (s, 1H, H-9_{bz}); 6.74-6.82 (m, 2H, Ar) ppm. ^{13}C NMR ($CDCl_3$, APT) δ: 23.09 (C-5_{cy}); 30.07 (C-6_{cy}); 32.40 (C-4_{cy}); 33.96 (C-5_{bz}); 35.71 (NCH_2); 37.54 (C-2_{cy}); 40.31(C-4_{bz}); 43.01 (C-1_{bz}); 48.22 (CH_2Ar); 50.79 (C-1_{cy}); 55.89 (OCH_3); 55.94 (OCH_3); 56.08 (OCH_3); 56.66 (C-3_{cy}); 111.50 (CH); 112.05 (CH); 113.31 (C-6_{bz}); 114.13 (C-9_{bz}); 120.54 (CH); 123.43 (C); 127.21 (C); 132.15 (C); 147.21 (C); 147.60 (C); 147.94 (C); 149.04 (C); 172.05 (CO) ppm. LR-MS (ESI) m/z calcd for $C_{28}H_{39}N_2O_5$ $[M+H]^+$: 483.3, found: 483.

The same procedure was used to prepare the following compounds:

cis **3-(3-{[(3,4-dimethoxyphenyl)methyl]amino}cyclohexyl)-7,8-dimethoxy-2,3,4,5-tetrahydro-1H-3-benzazepin-2-one 9b**: from**7** and 3,4-dimethoxybenzyl chloride **8b**,[2] oil, 26% yield. Eluent: abs EtOH/NH_4OH/ CH_2Cl_2/ Et_2O/ petroleum ether 180:10:360:360:900. From this mixture **3f** was also isolated.. Eluent: abs EtOH/NH_4OH/ CH_2Cl_2/ Et_2O/ petroleum ether 180:10:360:360:900.

9b: Oil, 26%.^{1}H-NMR ($CDCl_3$, δ): 1.01-1.12 (m, 1H); 1.25-1.48 (m, 3H); 1.67-1.83 (m, 3H); 1.90-2.04 (m, 2H); 2.70 (t, J = 10.8 Hz, 1H, H-3_{cy}); 2.99 (t, J=5.8 Hz, 2H, H-5_{bz}); 3.66 (t, J=5.8 Hz, 2H, H-4_{bz}); 3.74-3.90 (m, 16H, OCH_3 + H-1_{bz} + NCH_2Ar); 4.51 (m, 1H, H-1_{cy}); 6.51 (s, 1H, H-6_{bz}), 6.59 (s, 1H, H-9_{bz}), 6.80-6.84 (m, 3H, Ar) ppm. ESI-HRMS (m/z) calculated for $C_{27}H_{37}N_2O_5$ $[M+H]^+$ 469.2697, found 469.2689.

cis **3-(3-{bis[(3,4-dimethoxyphenyl)methyl]amino}cyclohexyl)-7,8-dimethoxy-2,3,4,5-tetrahydro-1H-3-benzazepin-2-one 3f**: from**7** and 3,4-dimethoxybenzyl chloride **8b**,[2] thick oil, 4%..^{1}H-NMR ($CDCl_3$, δ): 1.22-1.32 (m, 3H); 1.39-1.48 (m, 1H); 1.60-1.75 (m, 1H); 1.80-1.91 (m, 3H); 2.68-2.77 (m, 1H, H-3_{cy}); 2.92-2.96 (m, 2H, H-5_{bz}); 3.57 (s, 4H, $ArCH_2$); 3.63-3.67 (m, 2H, H-4_{bz}); 3.79-3.84 (m, 20H, OCH_3+H-1_{bz}); 4.34-4.47 (m, 1H, H-1_{cy}); 6.53 (s, 1H, H-6_{bz}); 6.59 (s, 1H, H-9_{bz}); 6.76 (d, J=8.0 Hz, 2H); 6.84 (d, J=8.0 Hz, 2H); 6.89 (s, 2H) ppm. ^{13}C-NMR-APT ($CDCl_3$, δ): 23.53 (CH_2); 27.67 (CH_2); 30.50 (CH_2); 33.19 (CH_2); 34.13 (CH_2); 40.52 (CH_2); 43.18 (CH_2); 51.86 (CH); 53.81 (CH_2); 55.88 (OCH_3); 56.04 (OCH_3); 57.18 (CH); 111.07 (CH); 111.63 (CH); 113.30 (CH); 114.15 (CH); 120.38 (CH); 123.51 (C); 127.28 (C); 133.43 (C); 147.25 (C); 148.01 (C); 148.99

(C); 172.03 (CO) ppm. ESI-HRMS (m/z) calculated for $C_{36}H_{47}N_2O_7$ $[M+H]^+$ 619.3378, found 619.3385.

cis **3-(3-{[3-(3,4-dimethoxyphenyl)propyl]amino}cyclohexyl)-7,8-dimethoxy-2,3,4,5-tetrahydro-1H-3-benzazepin-2-one 9c:** from **7** and 4-(3-iodopropyl)-1,2-dimethoxybenzene **8c**;[5] eluent: CH_2Cl_2/MeOH/NH_4OH 95:5:0.5. Oil, 17% yield.^{1}H-NMR ($CDCl_3$, δ): 1.02-1.11 (m, 1H); 1.24-1.43 (m, 4H); 1.68-1.98 (m, 6H); 2.55-2.71 (m, 5H, ArC$\underline{H}_2$CH_2C$\underline{H}_2$N + H-3_{cy}); 2.99 (t, J = 5.7 Hz, 2H, H-5_{bz}); 3.66 (t, J = 5.7 Hz, 2H, H-4_{bz}); 3.80-3.91 (m, 14H, OCH_3 + H-1_{bz}); 4.46-4.54 (m, 1H, H-1_{cy}); 6.52 (s, 1H, H-6_{bz}), 6.57 (s, 1H, H-9_{bz}); 6.68-6.70 (m, 2H); 6.76 (d, J = 6.0 Hz, 1H) ppm. LR-MS (ESI) m/z calcd for $C_{29}H_{41}N_2O_5$ $[M+H]^+$: 497.3, found: 497.

cis **3-(3-{[2-(3,4-dimethoxyphenoxy)ethyl]amino}cyclohexyl)-7,8-dimethoxy-2,3,4,5-tetrahydro-1H-3-benzazepin-2-one 9d**: from **7** and 4-(2-bromoethoxy)-1,2-dimethoxybenzene **8d**;[3] eluent: abs EtOH/NH_4OH/ CH_2Cl_2/ Et_2O/ petroleum ether 180:10:360:360:900. Oil, 23% yield. ^{1}H-NMR ($CDCl_3$, δ): 1.01-1.59 (m, 4H); 1.63-1.78 (m, 1H); 1.79-1.87 (m, 1H); 1.89-2.05 (m, 2H); 2.63-2.75 (m, 1H, H-3_{cy}); 2.98-3.00 (m, 4H, H-5_{bz}+CH_2N); 3.65-3.69 (m, 2H, H-4_{bz}); 3.75-3.85 (m, 14H, OCH_3 + H-1_{bz}); 4.01 (t, J = 4.8 Hz, 2H, CH_2O); 4.47-4.56 (m, 1H, H-1_{cy}); 6.37 (dd, J = 8.8 Hz, 2.4 Hz, 1H); 6.51 (s, 2H); 6.59 (s, 1H, H-9_{bz}); 6.75 (d, J = 8.8 Hz, 1H) ppm. LR-MS (ESI) m/z calcd for $C_{28}H_{39}N_2O_6$ $[M+H]^+$: 499.3, found: 499.

cis **3-(3-{[(2E)-3-(3,4-dimethoxyphenyl)prop-2-en-1-yl]amino}cyclohexyl)-7,8-dimethoxy-2,3,4,5-tetrahydro-1H-3-benzazepin-2-one 9e**: from **SB55** and 4-[(1E)-3-chloroprop-1-en-1-yl]-1,2-dimethoxybenzene **8e**;[4] eluent: CH_2Cl_2/MeOH/NH_4OH 95:5:0.5. Oil, 20% yield. ^{1}H NMR ($CDCl_3$) δ: 1.01-1.10 (m, 1H); 1.24-1.43 (m, 4H); 1.70-2.04 (m, 4H); 2.73 (t, H = 11.2 Hz, 1H, H-3_{cy}); 3.00 (t, J = 6.0 Hz, 2H, H-5_{bz}); 3.36-3.47 (m, 2H, NCH_2); 3.69 (t, J = 6.0 Hz, 2H, H-4_{bz}); 3.76-3.88 (m, 14H, OCH_3 + H-1_{bz}); 4.48-4.57 (m, 1H, H-1_{cy}); 6.14 (dt, J = 15.8, Hz, 6.6 Hz, 1H, CH=C); 6.45 (d, J = 15.8 Hz, 1H, CH=C); 6.51 (s, 1H, H-6_{bz}); 6.59 (s, 1H, H-9_{bz}); 6.79 (d, J = 8.0 Hz, 1H); 6.88 (dd, J = 8.0 Hz, 1.6 Hz, 1H); 6.93 (d, J = 1.6 Hz, 1H) ppm. LR-MS (ESI) m/z calcd for $C_{29}H_{39}N_2O_5$ $[M+H]^+$: 495.3, found: 495.

1.7. Synthesis of compounds 3a-e

cis **3-(3-{[2-(3,4-dimethoxyphenyl)ethyl](methyl)amino}cyclohexyl)-7,8-dimethoxy-2,3,4,5-tetrahydro-1H-3-benzazepin-2-one 3a** A solution of **8a** (0.31 mmol; 0.147 g), formic acid (5.12 mmol; 0.196 mL) and 37% aqueous formaldehyde (1.52 mmol; 0.115 mL) in absolute ethanol (2 mL) was heated at reflux for 15 hours. The reaction was monitored by TLC (eluent: dichloromethane-methanol, 85:15). The solvent was then removed under vacuum and the residue (yellow solid) treated with $NaHCO_3$ (saturated solution) and extracted with dichloromethane (3 × 15 mL). The organic layers were collected, dried (Na_2SO_4) and removed under vacuum to give a brown oil that was purified by gravity column chromatography. Eluting with dichloromethane-methanol-ammonia (95:5:0.5) afforded **3a** as a clear oil in 66% yields. ^{1}H NMR ($CDCl_3$) δ 1.17-1.28 (m, 1H, H-4_{cy}); 1.28-1.44 (m, 3H, H-6_{cy}+H-5_{cy}+H-2_{cy}); 1.72 (d, J=12.0 Hz, 1H, H-6_{cy}); 1.85-1.92 (m, 3H, H-4_{cy}+H-5_{cy}+H-2_{cy}); 2.38 (s, 3H, NCH_3); 2.62-2.75 (m, 5H, CH_2CH_2Ar+H-3_{cy}); 3.01 (t, J=5.6 Hz, 2H, H-5_{bz}); 3.69 (t, J=5.8 Hz, 2H, H-4_{bz}); 3.82 (s, 2H, H-1_{bz}), 3.84 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 4.51 (t, J=12.0 Hz, 1H, H-1_{cy}), 6.54 (s, 1H, H-6_{bz}), 6.61 (s, 1H, H-9_{bz}), 6.74-6.80 (m, 3H, Ar) ppm. ^{13}C NMR ($CDCl_3$, APT) δ 23.48 (C-5_{cy}); 28.39 (C-4_{cy}); 30.29 (C-6_{cy}); 32.24 (C-2_{cy}); 34.02(CH_2+C-5_{bz}); 37.82 (NCH_3); 40.49 (C-4_{bz}); 43.05 (C-1_{bz}); 51.68 (C-1_{cy}); 55.89 (OCH_3); 55.92 (OCH_3); 55.95 (OCH_3); 56.10 (CH_2); 61.44 (C-3_{cy}); 111.31 (CH); 112.14 (CH);

113.21 (C-6_{bz}); 114.05 (C-9_{bz}); 120.55 (CH); 123.41 (C); 127.15 (C); 132.98 (C); 147.16 (C); 147.38 (C); 147.91 (C); 148.86 (C); 172.01 (CO) ppm. ESI-HRMS (m/z) calculated for $C_{29}H_{41}N_2O_5$ $[M+H]^+$ 497.3010, found 497.3002.

The same procedure was used for the synthesis of the following compounds:

cis **3-(3-{[(3,4-dimethoxyphenyl)methyl](methyl)amino}cyclohexyl)-7,8-dimethoxy-2,3,4,5-tetrahydro-1H-3-benzazepin-2-one 3b**: from **9b**, eluent: abs EtOH/NH_4OH/ CH_2Cl_2/ Et_2O/ petroleum ether 180:10:360:360:900. Oil, 29%. ^{1}H-NMR ($CDCl_3$, δ): 1.19-1.50 (m, 4H); 1.69-1.72 (m, 1H); 1.85-1.96 (m, 3H); 2.20 (s, 3H, NCH_3); 2.62-2.68 (m, 1H, H-3_{cy}); 3.00 (t, J = 5.8 Hz, 2H, H-5_{bz}); 3.51 (d, J = 13Hz, 1H, ArC*H*HN); 3.56 (d, J = 13Hz, 1H, ArCH*H*N); 3.67-3.72 (m, 2H, H-4_{bz}); 3.81-3.87 (m, 14H, OCH_3+H-1_{bz}); 4.45-4.51 (m, 1H, H-1_{cy}); 6.53 (s, 1H, H-6_{bz}); 6.59 (s, 1H, H-9_{bz}); 6.77-6.81 (m, 2H, Ar); 6.89 (s, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$, APT) δ 23.55 (CH_2); 27.97 (CH_2); 30.41 (CH_2); 32.62 (CH_2); 34.16 (CH_2); 37.45 (NCH_3); 40.63 (CH_2); 43.19 (NCH_3); 51.86 (CH); 56.04 (OCH_3); 58.10 ($ArCH_2$); 61.25 (CH); 111.03 (CH); 112.05 (CH); 113.34 (CH); 114.19 (CH); 121.02 (CH); 123.54 (C); 127.29 (C); 147.28 (C); 148.04 (C); 149.15 (C); 172.13 (CO) ppm. ESI-HRMS (m/z) calculated for $C_{28}H_{39}N_2O_5$ $[M+H]^+$ 483.2853, found 483.2844.

cis **3-(3-{[3-(3,4-dimethoxyphenyl)propyl](methyl)amino}cyclohexyl)-7,8-dimethoxy-2,3,4,5-tetrahydro-1H-3-benzazepin-2-one 3c**: from **9c**, oil, 89%. ^{1}H-NMR ($CDCl_3$, δ): 1.10-1.39 (m, 4H); 1.66-1.85 (m, 6H); 2.24 (s, 3H, NCH_3); 2.38-2.55 (m, 5H); 2.98 (t, J = 5.6 Hz, 2H, H-5_{bz}); 3.62-3.70(m, 2H, H-4_{bz}); 3.79-3.90 (m, 14H, OCH_3+H-1_{bz}); 4.43-4.49 (m, 1H, H-1_{cy}); 6.51 (s, 1H, H-6_{bz}); 6.58 (s, 1H, H-9_{bz}); 6.68-6.72 (m, 2H); 6.77 (d, J = 8.8 Hz, 1H) ppm. ^{13}C NMR ($CDCl_3$, APT) δ 23.56 (CH_2); 28.39 (CH_2); 29.63 (CH_2); 30.41 (CH_2); 32.26 (CH_2); 33.19 (CH_2); 34.08 (CH_2); 37.66 (NCH_3); 40.48 (CH_2); 43.11 (CH_2); 51.71 (CH); 53.46 (CH_2); 55.86 (OCH_3); 55.97 (OCH_3); 61.40 (CH); 111.23 (CH); 111.73 (CH); 113.20 (CH); 114.04 (CH); 120.18 (CH); 123.45 (C); 127.21 (C); 134.90 (C); 147.15 (C); 147.91 (C); 148.80 (C); 172.06 (CO) ppm. ESI-HRMS (m/z) calculated for $C_{30}H_{43}N_2O_5$ $[M+H]^+$ 511.3166, found 511.3163.

cis **3-(3-{[2-(3,4-dimethoxyphenoxy)ethyl](methyl)amino}cyclohexyl)-7,8-dimethoxy-2,3,4,5-tetrahydro-1H-3-benzazepin-2- one 3d**: from **9d**, eluent: abs EtOH/NH_4OH/ CH_2Cl_2/ Et_2O/ petroleum ether 180:10:360:360:900. Oil, 63%. ^{1}H-NMR ($CDCl_3$, δ): 1.12-150 (m, 4H); 1.72-2.01 (m, 4H); 2.44 (s, 3H, NCH_3); 2.69-2.79 (m, 1H, H-3_{cy}); 2.90-3.01 (m, 4H, NCH_2+H-5_{bz}); 3.64-3.71 (m, 2H, H-4_{bz}); 3.80-3.91 (m, 14H, OCH_3+H-1_{bz}); 4.05 (t, J = 5.8 Hz, 2H, CH_2O); 4.42-4.54 (m, 1H, H-1_{bz}); 6.38 (dd, J = 8.8 Hz, 2.8 Hz, 1H); 6.50-6.52 (m, 1H); 6.59 (s, 1H); 6.75 (d, J = 8.8 Hz, 1H) ppm. ^{13}C NMR ($CDCl_3$, APT) δ 23.43 (CH_2); 28.10 (CH_2); 30.21 (CH_2); 32.21 (CH_2); 34.10 (CH_2); 38.65 (NCH_3); 40.69 (CH_2); 43.12 (CH_2); 51.69 (CH); 52.55 (CH_2); 56.03 (OCH_3); 56.54 (OCH_3); 65.02 (CH); 66.44 (OCH_2); 100.97 (CH); 103.91 (CH); 111.93 (CH); 113.29 (CH); 114.14 (CH); 123.43 (C); 127.21 (C); 143.73 (C); 147.27 (C); 148.03 (C); 149.99 (C); 153.25 (C); 172.20 (CO) ppm. ESI-HRMS (m/z) calculated for $C_{29}H_{41}N_2O_6$ $[M+H]^+$ 513.2959, found 513.2967.

cis **3-(3-{[(2E)-3-(3,4-dimethoxyphenyl)prop-2-en-1-yl](methyl)amino}cyclohexyl)-7,8-dimethoxy-2,3,4,5-tetrahydro-1H-3-benzazepin-2-one 3e**: from **9e**, oil, 90%. ^{1}H-NMR ($CDCl_3$, δ): 1.20-1.48 m, 4H); 1.70-1.96 (m, 4H); 2.29 (s, 3H, NCH_3); 2.62-2.67 (m, 1H, H-3_{cy}); 3.02 (t, J = 5.6 Hz, 2H, H-5_{bz}); 3.21-3.32 (m, 2H, CH_2N); 3.70 (t, J = 5.6 Hz, 2H, H-4_{bz}); 3.78-3.89 (m, 14H, OCH_3+H-1_{bz}); 4.47-4.53 (m, 1H, H-1_{cy}); 6.10 (dt, J = 15.6 Hz, 6.8 Hz, 1H, C*H*=CH); 6.43 (d, J = 15.6 Hz, 1H, CH=C*H*); 6.53 (s, 1H, H-6_{bz}); 6.60 (s, 1H, H-9_{bz}); 6.80 (d, J = 8.2 Hz, 1H, Ar); 6.89 (d, J = 8.2 Hz, 1H, Ar); 6.95 (s, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$, APT) δ 23.73 (CH_2); 28.58 (CH_2); 30.56 (CH_2); 32.95 (CH_2); 34.35 (CH_2); 37.79 (NCH_3); 40.80 (CH_2); 43.39 (CH_2); 51.91 (CH); 56.13

(OCH_3); 56.24 (OCH_3); 56.95 (CH_2); 61.36 (CH); 108.86 (CH); 111.36 (CH); 113.45 (CH); 114.31 (CH); 119.88 (CH); 123.70 (C); 127.45 (C); 130.07 (CH); 130.35 (C); 132.50 (CH); 147.44 (C); 148.19 (C); 149.03 (C); 149.33 (C); 172.37 (CO) ppm. ESI-HRMS (m/z) calculated for $C_{30}H_{41}N_2O_5$ $[M+H]^+$ 509.3010, found 509.3017.

1.8. Synthesis of compounds 3g

cis **5-(7,8-dimethoxy-2-oxo-1,2-dihydro-3H-benzo[d]azepin-3-yl)cyclohex-3-ene-1-carboxylic acid**

This compound was prepared starting from *cis*-**4**, using the same procedure applied for **3a**. Therefore, *cis*-**4** was hydrolyzed using NaOH as reported for **6**, yielding 5-(7,8-dimethoxy-2-oxo-2,3-dihydro-1H-3-benzazepin-3-yl)cyclohex-3-ene-1-carboxylic acid as a solid, mp 61.62°C, 95%. ^{1}H NMR ($CDCl_3$) δ 1.56 (dd, J = 12.4 Hz, 11.2 Hz, 1H); 2.16-2.43 (m, 3H); 2.72-2.86 (m, 1H, H-1_{cy}); 3.39 (d, J = 12.4 Hz, 1H, H-1_{bz}); 3.52 (d, J = 12.4 Hz, 1H, H-1_{bz}); 3.87 (s, 3H, OCH_3); 3.89 (s, 3H, OCH_3); 5.32-5.41 (m, 1H, H-5_{cy}); 5.51 (d, J = 10.0 Hz, 1H, H-4_{cy}); 5.93-6.00 (m, 1H, H-3_{cy}); 6.19 (d, J = 9.2 Hz, 1H, H-4_{bz}); 6.40 (d, J = 9.2 Hz, 1H, H-5_{bz}); 6.72 (s, 1H, H-6_{bz}); 6.80 (s, 1H, H-9_{bz}) ppm.

cis **3-(5-aminocyclohex-2-en-1-yl)-7,8-dimethoxy-2,3-dihydro-1H-3-benzazepin-2-one** was prepared as reported for **7** following method B; it was obtained as white solid, m.p. 56-58 °C, 59%. ^{1}H NMR ($CDCl_3$) δ 1.32 (q, J = 11.4 Hz, 1H); 1.57 (bs, 1H, NH_2); 1.77-1.88 (m, 1H); 1.92-1.98 (m, 1H); 2.24-2.34 (m, 1H); 3.03-3.15 (m, 1H, H-5_{cy}); 3.37 (d, J = 12.4 Hz, 1H, H-1_{bz}); 3.48 (d, J = 12.4 Hz, 1H, H-1_{bz}); 3.86 (s, 3H, OCH_3); 3.88 (s, 3H, OCH_3); 5.29-5.38 (m, 1H, H-1_{cy}); 5.43 (d, J = 9.6 Hz, 1H, H-4_{cy}); 5.83-5.92 (m, 1H, H-3_{cy}); 6.19 (d, J = 9.4 Hz, 1H, H-4_{bz}); 6.37 (d, J = 9.2 Hz, 1H, H-5_{bz}); 6.71 (s, 1H, H-6_{bz}); 6.77 (s, 1H, H-9_{bz}) ppm. ESI-HRMS $C_{18}H_{23}O_3N_2$ 315.1706 $[M+1]^+$.

cis **3-(5-{[2-(3,4-dimethoxyphenyl)ethyl]amino}cyclohex-2-en-1-yl)-7,8-dimethoxy-2,3-dihydro-1H-3-benzazepin-2-one** was prepared as reported for **8a**, and it was obtained as oil in 65% yield. ^{1}H NMR ($CDCl_3$) δ 1.32 (q, J = 11.6 Hz, 1H); 1.79-1.84 (m, 1H); 1.97-2.04 (m, 1H); 2.23-2.33 (m, 1H); 2.62-2.74 (m, 2H, CH_2Ar); 2.78-2.89 (m, 3H, CH_2N+H-5_{cy}); 3.34 (d, J = 12.4 Hz, 1H, H-1_{bz}); 3.45 (d, J = 12.4 Hz, 1H, H-1_{bz}); 3.80 (s, 3H, OCH_3); 3.81 (s, 3H, OCH_3); 3.84 (s, 3H, OCH_3); 3.85 (s, 3H, OCH_3); 5.25-5.33 (m, 1H, H-1_{cy}); 5.42 (d, J = 10.0 Hz, 1H, H-4_{cy}); 5.82-5.89 (m, 1H, H-3_{cy}); 6.16 (d, J = 9.2 Hz, 1H, H-4_{bz}); 6.34 (d, J = 9.2 Hz, 1H, H-5_{bz}); 6.65-6.69 (m, 3H, Ar); 6.73-6.77 (m, 2H, Ar) ppm.

cis **3g** was finally prepared as reported for **3a**, and it was obtained as solid, m.p. 60-63°C; 51%.^{1}H NMR ($CDCl_3$) δ 1.34 (q, J = 11.6 Hz, 1H); 1.99-2.06 (m, 1H); 2.08-2.22 (m, 2H); 2.31 (s, 3H. NCH_3); 2.55-2.71 (m, 4H, NCH_2CH_2Ar); 2.84-2.92 (m, 1H, H-5_{cy}); 3.35 (d, J = 12.4 Hz, 1H, H-1_{bz}); 3.48 (d, J = 12.4 Hz, 1H, H-1_{bz}); 3.82 (s, 3H, OCH_3); 3.83 (s, 3H, OCH_3); 3.85 (s, 3H, OCH_3); 3.87 (s, 3H, OCH_3); 5.29-5.37 (m, 1H, H-1_{cy}); 5.41 (d, J = 10.0 Hz, 1H, H-4_{cy}); 5.88-5.94 (m, 1H, H-3_{cy}); 6.18 (d, J = 9.4 Hz, 1H, H-4_{bz}); 6.35 (d, J = 9.4 Hz, 1H, H-5_{bz}); 6.68-6.71 (m, 3H, Ar); 6.73-6.79 (m, 2H, Ar) ppm. ^{13}C NMR ($CDCl_3$, APT) δ 28.09 (CH_2); 30.07 (CH_2); 34.19 (CH_2); 37.70 (NCH_3); 43.50 (CH_2); 52.64 (CH); 55.74 (CH_2); 55.86 (OCH_3); 55.93 (OCH_3); 55.98 (OCH_3); 58.16 (CH); 109.54 (CH); 111.15 (CH); 111.25 (CH); 112.06 (CH); 117.72 (CH); 120.55 (CH); 124.72 (C); 124.75 (CH); 126.53 (C); 127.76 (CH); 130.13 (CH); 132.94 (C); 147.34 (C); 148.03 (C); 148.81 (C); 149.83 (C); 167.41 (CO) ppm. ESI-HRMS (m/z) calculated for $C_{29}H_{37}N_2O_5$ $[M+H]^+$ 493.2697, found 493.2701.

1.9 4-(3-iodopropyl)-1,2-dimethoxybenzene 8c [5]

A solution of ethyl 3-(3,4-dimethoxyphenyl)propanoate (prepared from the commercially available acid under standard conditions; 0.9 g, 3.8 mmol) in anhydrous THF (5 mL) was added dropwise to a suspension of $LiAlH_4$ (0.29 g, 7.6 mmol) in anhydrous THF (5 mL), kept at -15°C. After stirring at room temperature for 5 hrs, ice was added, the solvent was removed and the residue partitioned between H_2O and ethyl acetate. Dying (Na_2SO_4) and removal of solvent gave 0.69 g of the alcohol as an oil (93% yield). ^{1}H-NMR ($CDCl_3$, δ): 1.78-1.85 (m, 2H, CH_2); 2.53 (bs, 1H, OH); 2.59 (t, J=7.8 Hz, 2H, CH_2); 3.60 (t, J=6.6 Hz, 2H, CH_2); 3.79 (s, 3H, OCH_3); 3.80 (s, 3H, OCH_3); 6.66-6.78 (m, 3H, Ar) ppm. A solution of this compound, Et_3N (1.1 eq) and $SOCl_2$ (1.5 eq) in $CHCl_3$ was lest stirring at room T for 24 hrs, then the solvent was removed under vacuum and the residue partitioned between H_2O and CH_2Cl_2. Dying (Na_2SO_4) and removal of solvent gave 0.67 g of the chloride[9] as an oil (89% yield). ^{1}H-NMR ($CDCl_3$, δ): 1.98-2.05 (m, 2H, CH_2); 2.68 (t, J=7.4 Hz, 2H, CH_2); 3.48 (t, J=6.6 Hz, CH_2Cl); 3.81 (s, 3H, OCH_3); 3.83 (s, 3H, OCH_3); 6.68-6.78 (m, 3H, Ar) ppm. The chloride was dissolved in acetone (30 mL) and NaI (3 eq) was added. The mixture was heated under reflux for 48 hr, then the solvent was removed under vacuum and the residue partitioned between H_2O and CH_2Cl_2. Drying (Na_2SO_4) and removal of solvent gave 0.86 g of the iodide[5] as an oil (90% yield). ^{1}H-NMR ($CDCl_3$, δ): 2.05-2.13 (m, 2H, CH_2); 2.67 (t, J=7.2 Hz, 2H, CH_2); 3.16 (t, J=6.8 Hz, 2H, CH_2); 3.85 (s, 3H, OCH_3); 3.87 (s, 3H, OCH_3); 6.72-6.80 (m, 3H, Ar) ppm.

1.10 HPLC Separation of the enantiomers of 3a

The separation of *rac*-**3a** was carried out with a Perkin-Elmer series 200 semi-preparative liquid chromatography system composed by autosampler, quaternary pump, UV detector and a Biorad fraction collector. The resolution of the enantiomers were performed with Phenomenex Lux Amylose-2 column 250 mm length, 4.6 mm internal diameter and 5 μm particle size, in isocratic elution. The mobile phase used was a mixture of methanol:iso-propanol 90:10 (v/v) at constant flow of 1.5 mL min^{-1}. The injection volume was 100 μL, the column temperature kept at 40°C and UV detection at 220 nm. By using these chromatographic conditions, the elution of the enantiomers allows their collection in two different fractions. Figure S2 shows the typical chromatogram of *rac*-**3a**.

The fractions corresponding of the two enantiomers were collected in different reservoirs, the solvent was removed under vacuum and the residues were weighted. About 10 mg of each enantiomer was recovered, then they were characterized by through their enantiomeric excess and optical purity.

The enantiomeric excess (ee) was carried out in the same condition reported above except the column length, that was 50 mm. The chromatographic profiles of ee analysis of the enantiomers were reported in Figure S3; the enantiomeric excess was determined >95%

(+)-**3a** (+EC18) (first eluted isomer): $[\alpha]^{20}_D$ ($CHCl_3$, c=1) +17.66°

(-)-**3a** (-EC18) (second eluted isomer): $[\alpha]^{20}_D$ ($CHCl_3$, c=1) -19.08°

2. PHARMACOLOGY

2.1 Electrophysiological experiments on HEK-293 cells.

Cell culture and isolation, patch-clamp recordings, data analysis and statistics were performed as reported in ref. 10,11

2.2 Negative chronotropic activity

The negative chronotropic activity of compound **3a** was determined as previously reported.[12]

2.3 Electrophysiological experiments on dog papillary muscle

All experiments were carried out in compliance with the Guide for the Care and Use of Laboratory Animals (USA NIH publication No 85-23, revised 1985). The protocols were approved by the Review Board of the Committee on Animal Research of the University of Szeged (54/1999 OEj). Ventricular muscles were obtained from the right ventricle of hearts removed through a right lateral thoracotomy from anesthetized (thiopental 30 mg/kg *i.v.*) mongrel dogs of either sex weighing 10 - 15 kg. The preparations were placed in a tissue bath and allowed to equilibrate for at least 2 h while superfused (flow rate 4-5 ml/min) with Locke's solution containing (in mM): NaCl 120, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 22, and glucose 11. The pH of this solution was 7.40 to 7.45 when gassed with 95% O_2 and 5% CO_2 at 37°C. During the equilibration period, the ventricular muscle tissues were stimulated at a basic cycle length of 1000 ms. Electrical pulses of 2 ms in duration and twice diastolic threshold in intensity (S_1) were delivered to the preparations through bipolar platinum electrodes. Transmembrane potentials were recorded with the use of glass capillary microelectrodes filled with 3 M KCl (tip resistance: 5 to 15 MΩ). The microelectrodes were coupled through an Ag-AgCl junction to the input of a high-impedance, capacitance-neutralizing amplifier (Experimetria 2009). Intracellular recordings were displayed on a storage oscilloscope (Hitachi V-555) and led to a computer system (APES) designed for on-line determination of the following parameters: resting membrane potential, action potettial amplitude, action potential duration at 50% and 90% repolarization and the maximum rate of rise of the action potential upstroke (V_{max}). Stimulation with a constant cycle length of 1000 ms (ventricular muscles) was applied in the course of the experiments. Control recordings were obtained after equilibrium period. The effects of EC18 was determined at the given concentrations, with recordings started 25 minutes after the addition of each concentration of the drug in a cumulative manner. For all experiments **3a** (EC18) was dissolved in distilled water at stock solution concentration of 1 or 10 mM.

Results were analyzed by using Student's t-test for paired and unpaired data. Differences were considered significant when $p < 0.05$. Data are expressed as mean ± S.E.M. (standard error of the mean).

2.4 Electrophysiological experiments in thalamic neurons

Whole-cell patch-clamp recordings were performed on thalamocortical relay (TC) neurons in brain slices[13] or following acute isolation[14] as described before. Experiments were performed on C57BL/6J mice ranging in age from postnatal day P16 to 35. A block of tissue containing the thalamus was removed from the brain and submerged in ice-cold aerated (O_2) saline containing (in mM): sucrose, 200; PIPES, 20; KCl, 2.5; NaH_2PO_4, 1.25; $MgSO_4$, 10; $CaCl_2$, 0.5; dextrose, 10; pH 7.35, with NaOH.

For recordings in brain slices coronal sections (250 - 300 µm thickness) containing the ventrobasal thalamic complex (VB) were prepared as on a vibratome. Slices were transferred to a holding chamber and kept submerged (at 30°C for 30 min, thereafter at room temperature) in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 125; KCl, 2.5; NaH_2PO_4, 1.25; $NaHCO_3$, 24; $MgSO_4$, 2; $CaCl_2$, 2; dextrose, 10; pH adjusted to 7.35 by bubbling with carbogen (95% O_2 and 5% CO_2 gas mixture). Recordings were done on visually identified TC neurons of the VB in a solution containing (in mM): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.25; HEPES, 30; $MgSO_4$, 2; $CaCl_2$, 2; dextrose, 10; pH 7.25 adjusted with HCl. In order to block inward rectifying K^+ and K_{2P} channels, 0.5 mM $BaCl_2$ was added to the solution. Whole-cell recordings were made from the soma of TC neurons at 30 - 32ºC. Membrane currents were measured with glass microelectrodes pulled from borosilicate glass capillaries (GC150T-10; Clark Electromedical Instruments, Pangbourne, UK) filled with (in mM): K-gluconate, 95; K_3-citrate, 20; NaCl, 10; HEPES, 10; $MgCl_2$, 1; $CaCl_2$, 0.5; BAPTA, 3; Mg-ATP, 3; Na_2-GTP, 0.5. The internal solution was set to a pH of 7.25 with KOH and an osmolality of 295 mOsm/kg. Patch electrodes were connected to an EPC-10 amplifier (HEKA Elektronik, Lamprecht, Germany) via a chlorinated silver wire. Electrode resistances were in the range of 2.5-3.5 MΩ, with access resistances in the range of 8-20 MΩ. Series resistance compensation of > 50% was routinely applied. Voltage-clamp experiments were controlled by the software PatchMaster (HEKA Elektronik) operating on an IBM-compatible personal computer. EC18 (**3a**) hydrochloride was dissolved as stock solution in water and appropriate aliquots were added to ACSF. The compound was applied for 20 min by bath application in brain slices. I_h was elicited from a holding potential of -40 by a hyperpolarizing step to -130 mV. The dose-response relationship of I_h amplitude reduction was estimated by fitting a sigmoidal function to the data points (0.1 µM, n = 2; 1 µM, n = 2; 10 µM, n = 2; 20 µM, n = 3; 30 µM, n = 2; 100 µM, n = 3): $Y = A1 + \{(A2 – A1) / (1 + \exp((\log x0 - x)p))\}$, with A1 and A2 the bottom and top asymptote, respectively, x0 the half maximal inhibitory concentration (EC_{50}) and p the Hill slope.

For recordings in isolated neurons tissue containing the dorsal part of the lateral geniculate nucleus dLGN) was transferred to a spinner flask and incubated for 25-30 min at 30°C in an oxygenated solution containing trypsin (0.5-1 mg/ml, Sigma) and (mM): 120 NaCl, 5 KCl, 3 $MgCl_2$, 1 $CaCl_2$, 20 PIPES, 25 dextrose, pH 7.35. Single neurons were obtained by trituration. Whole-cell recordings were performed on identified dLGN TC neurons at room temperature. Borosilicate glass pipettes with a resistance of 3-5 MΩ were used; typical access resistance was in the range of 4-10 MΩ. Series resistance compensation of >30% was routinely applied. Voltage-clamp experiments were controlled by the software PatchMaster (HEKA Elektronik) operating on an IBM-compatible personal computer and outward currents were evoked by depolarizing voltage steps. For voltage clamp recordings the extracellular solution contained (mM): 140 NaCl, 2 KCl, 10 HEPES, 10 dextrose, 3 $MgCl_2$, 1 $CaCl_2$, 0.001 TTX, 0.15 $CdCl_2$ (pH 7.35 and 305 mOsm). The intracellular solution contained (mM): 85 K-gluconate, 10 K_3citrate, 10 NaCl, 10 KCl, 3 K-BAPTA, 0.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 3 MgATP, 0.5 Na_2GTP, 15 phosphocreatin; pH 7.25 and 295 mOsm. A custom-made multibarreled laminar flow perfusion system which allowed rapid exchange of solutions and the application of drugs was installed close to the recorded neuron. EC18 was dissolved as stock solution in water and appropriate aliquots were added to extracellular solution. Outward currents were evoked by depolarizing voltage-steps from -48 to +32 mV (1 s duration; holding potential -58 mV; conditioning potential -108 mV for 1 s). The blocker-sensitive component was calculated by graphical subtraction of the currents recorded in the presence and absence of EC18. The current-voltage relationship of the late current

component was generated by determining current amplitudes shortly before stepping back to the holding potential and plotting mean values against the depolarizing step potential.

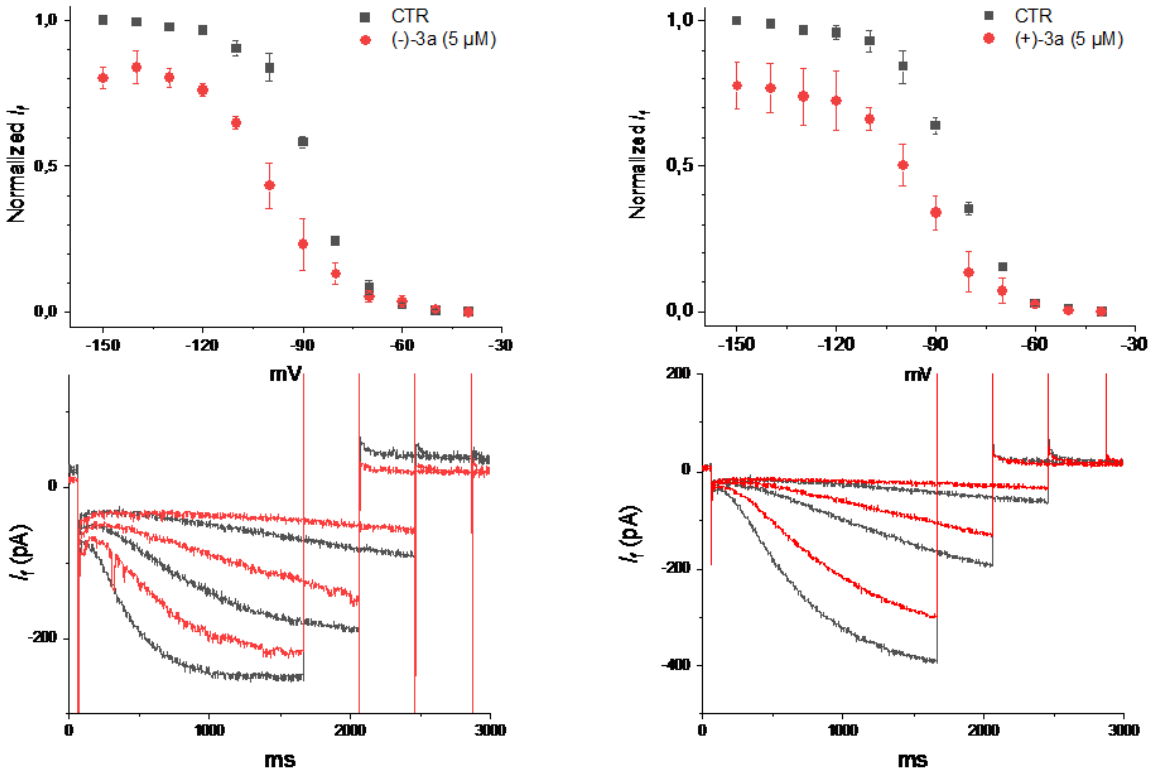

Figure S1. **Effect of 3a enantiomers on recombinant HCN4 channel**. Top plots represent HCN4-mediated current values, expressed as normalized current (sem), as a function of voltage potentials before (black dots) and after (red dots) application of 5µM (–)-**3a** (left) or (+)-**3a** (right). Bottom traces are typical examples of HCN4 mediated current evoked at -80/-120 mV before (black lines) and after (red lines) (–)-**3a** (left) or (+)-**3a** (right).

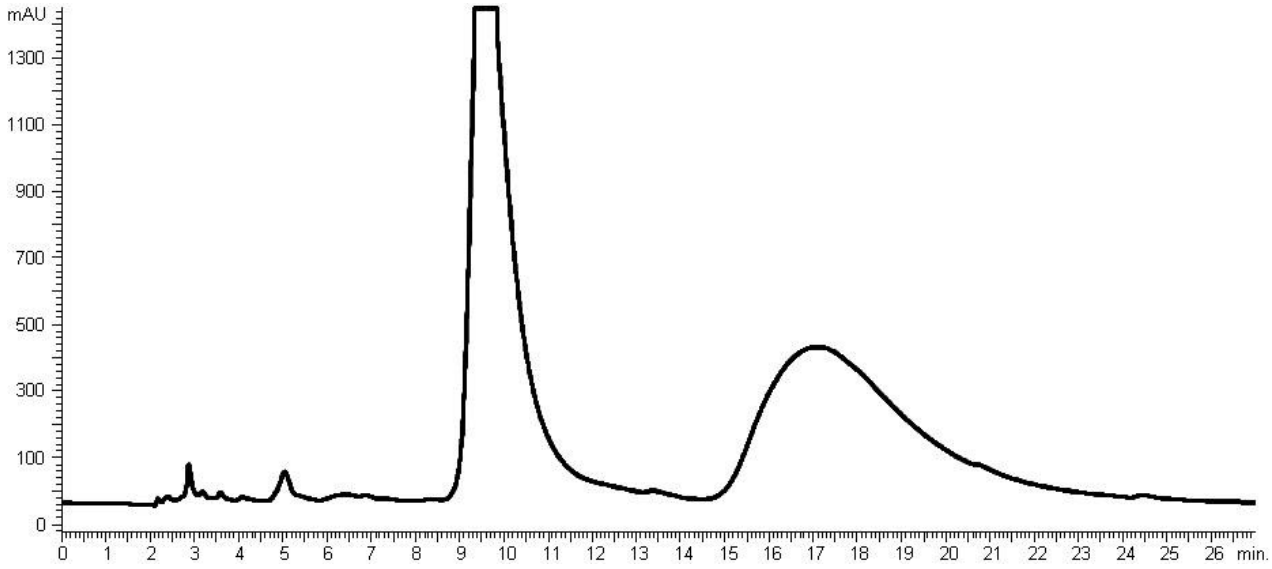

Figure S2: LC-UV chromatographic profile of *rac*-**3a**.

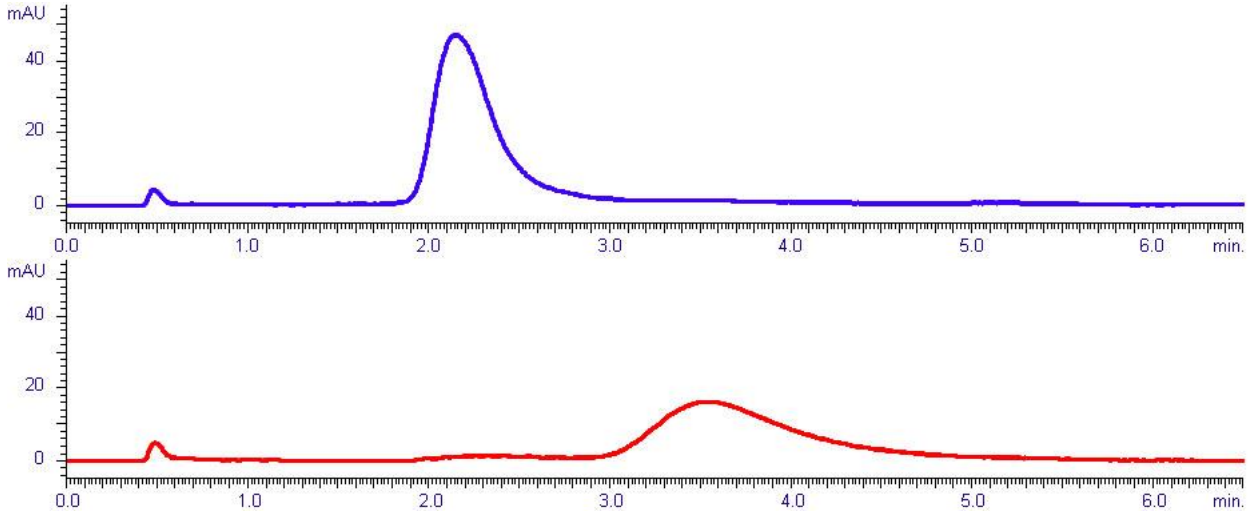

Figure S3: LC-UV chromatographic profiles and ee of the collected 3a enantiomers .

Table S1. The electrophysiological effects of **3a** (EC18) in dog (n=5) ventricular muscle preparations at stimulation cycle length of 1000 ms.

	Control	**EC18 - 1 µM**	**EC18 - 10 µM**
CT (ms)	4.8 ± 0.60	4.7 ± 0.59	5.0 ± 0.57
MDP (mV)	-83.8 ± 2.28	-84.0 ± 2.25	-83.0 ± 2.05
APA (mV)	104.1 ± 2.7	103.1 ± 1.62	102.3 ± 1.42
Vmax (Vs^{-1})	244.6 ± 30.87	232.9 ± 33.6	140.6 ± 20.24*
APD90 (ms)	212.5 ± 16.25	204.9 ± 12.5	220.2 ± 13.57
APD75 (ms)	199.7 ± 16.87	191.9 ± 13.40	206.0 ± 14.50
APD50 (ms)	176.0 ± 16.36	168.3 ± 13.03	179.9 ± 14.85
APD25 (ms)	131.7 ± 13.1	121.6 ± 10.06	125.1 ± 9.35
APD10 (ms)	69.9 ± 10.84	58.6 ± 9.73	55.7 ± 14.1

Values are means ± SEM. CT, conduction time; MDP, maximum diastolic potential; APA, action potential amplitude; Vmax, maximum rising velocity of the action potential upstroke.
APD90, action potential duration at 90% of repolarization; APD75, action potential duration at 75% of repolarization; APD50, action potential duration at 50% of repolarization; APD25, action potential duration at 25% of repolarization; APD10, action potential duration at 10% of repolarization.
*$p<0.05$ vs control, n=5 (preparations obtained from different animals), cumulative drug application.

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