Supporting Information

ACS Chemical Neuroscience

Pharmacological characterization of [^{3}H]ATPCA as a substrate for studying the functional role of the betaine/GABA transporter 1 and the creatine transporter

Anas Al-Khawaja,[†] Anne S. Haugaard,[†] Ales Marek,[‡] Rebekka Löffler,[†] Louise Thiesen,[†] Mònica Santiveri,[†] Maria Damgaard,[†] Christoffer Bundgaard,[§] Bente Frølund,[†] and Petrine Wellendorph[*,†]

[†]Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, 2100 Copenhagen, Denmark

[‡]Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague 6, Czech Republic, Flemingovo nam 542/2, 16610

[§]*Discovery Chemistry and DMPK, H. Lundbeck A/S, 2500 Valby, Denmark*

Pharmacology

$GABA_B$ receptor binding

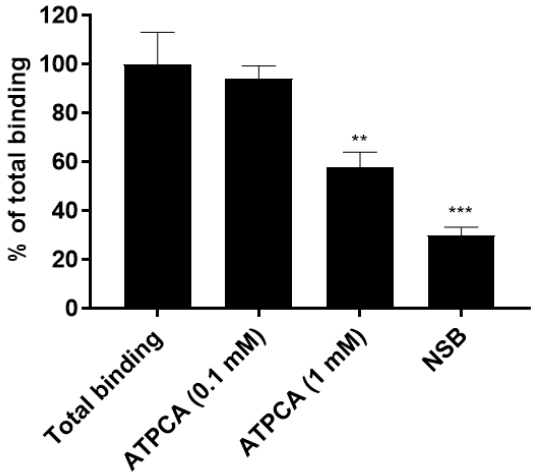

Figure S1. The ability of ATPCA to displace $GABA_B$ receptor binding in rat cortical membranes using [^{3}H]GABA (5 nM) and isoguvacine (40 μM) to saturate $GABA_A$ receptor binding. Non-specific binding (NSB) was determined in the presence of baclofen (100 μM). Data are normalized to total binding and are means ± S.E.M. of two independent experiments performed in triplicates. All results were compared to total binding (** P<0.01; *** P<0.001; One-way ANOVA followed by Dunnett's multiple comparison test).

Brain penetration of ATPCA

The brain-to-plasma (B/P) distribution ratio of ATPCA was investigated acutely in mice 30, 60 and 120 min after subcutaneous administration of 10 mg/kg and was found to be 0.05, 0.12 and 0.17, respectively. Whereas the plasma concentration of ATPCA was decreasing with time (~1,000, ~450 and 340 ng/mL at 30, 60 and 120 min, respectively), the brain concentration was constant (~53 ng/g). Combining the B/P ratios with the free fractions in brain and plasma, the estimated unbound distribution ratios were 0.02, 0.06 and 0.08 suggesting a restricted brain penetrance of ATPCA under these conditions. For comparison, we also investigated the brain penetration of **1** after oral administration of 10 mg/kg and did not detect it in the brain despite achieving plasma levels of 2,200 ng/ml at this dose.

Chemistry

General experimental section

The tritiation reaction was performed on a custom-designed tritium manifold system manufactured by RC Tritec AG, Switzerland. Tritium gas stored as uranium tritide on a uranium bed was released by heating to approximately 500 °C. The deuterium experiment was carried out on a deuterium manifold system (RC Tritec AG) equipped with pressure bottle containing gaseous deuterium (purity ≥99.9, enrichment ≥99.8%; Linde AG). The ^{1}H and ^{3}H NMR spectra were recorded at 300 MHz and 320 MHz, respectively, with a Bruker Avance II 300 MHz instrument at 25 °C (solvent indicated in parentheses). HPLC analysis and separation of deuterium samples were performed using a Knauer smartline instrument, pump 1000, UV detector 2500. HPLC analysis and separation of deuterium and tritium samples were performed using a Waters 1524, binary pump; Waters 2487, dual absorbance detector, equipped with Bioscan Hidex liquid scintillation counter. HPLC grade water obtained by purification using an Aurium 611 UV system from Sartorius, Germany was used. Liquid scintillation counting was performed by a Perkin-Elmer Tri-Carb 2900TR Liquid Scintillation Counter (LSC) in a Zinsser Quicksafe A cocktail. Mass spectra of labeled compounds was obtained by a Bruker Daltonics esquire 4000 electrospray ion-trap system by either direct injection or by a Merck Hitachi HPLC for LC-MS analysis. The HR-MS spectra were obtained in the ESI mode either on a Q-Tof micro from Waters or on an LTQ Orbitrap XL from Thermo Fisher Scientific. LC-MS (System B) was performed using a Agilent 1100 HPLC systems with a XBridge 3.5 µm C-18 column (100 x 4.60 mm) using gradient elution from buffer A (H_2O:CH_3CN:HCOOH, 95:5:0.1) to buffer B (H_2O:CH_3CN: HCOOH, 5:95:0.05) over 8 min flow rate: 0.5 mL/min, coupled to an Hewlett Packard 1100 series mass spectrometer with an electrospray ionization source.

Optimized deuteration conditions for isotopic labelling of ATPCA

[^{2}H]-2-Amino-1,4,5,6-tetrahydro-4,5,6-pyrimidine-5-carboxylic acid ([^{2}H]ATPCA). 2-Aminopyrimidine-5-carboxylic acid (20 mg, 143 μmol), Pd/C [30%] (28 mg) and dry DMF (1 mL) were placed in a round-bottomed deuteration flask (2 mL). The flask was mounted on the deuterium manifold, and the reaction mixture was degassed by three successive freeze-thaw cycles (liquid nitrogen, vacuum of oil pump). The reaction mixture was stirred under D_2 (1045 mbar) for 4 hr at room temperature. The mixture was cooled by liquid nitrogen, and the D_2 was disposed of under vacuum. The reaction mixture was filtered through a 0.45 µm PTFE syringe filter, and the

deuteration flask and filter were washed by three 1 mL portions of DMF. A sample of reaction mixture was analyzed on the HPLC showing almost quantitative conversion of starting material, the expected product as the only one product in the reaction mixture. The DMF was evaporated on the Centrivap resulting in the crude [^{2}H]ATPCA as a white solid. The crude product was used for ^{1}H NMR and MS analysis without other purification. ESI-HRMS data determined enrichment of deuterium (0:1:2:3 in a approx. ratio of 70:100:60:15 based on the intensities of appropriate signals). [^{2}H]ATPCA, MS (m/z): 134.1 [M-1]. [^{2}H]ATPCA-1, HRMS *m/z* (ESI+) found: 145.08271 $[M+1]^+$; $C_5H_9DN_3O_2$ requires *M*, 145.08303.

Representative MS-spectrum and NMR spectra

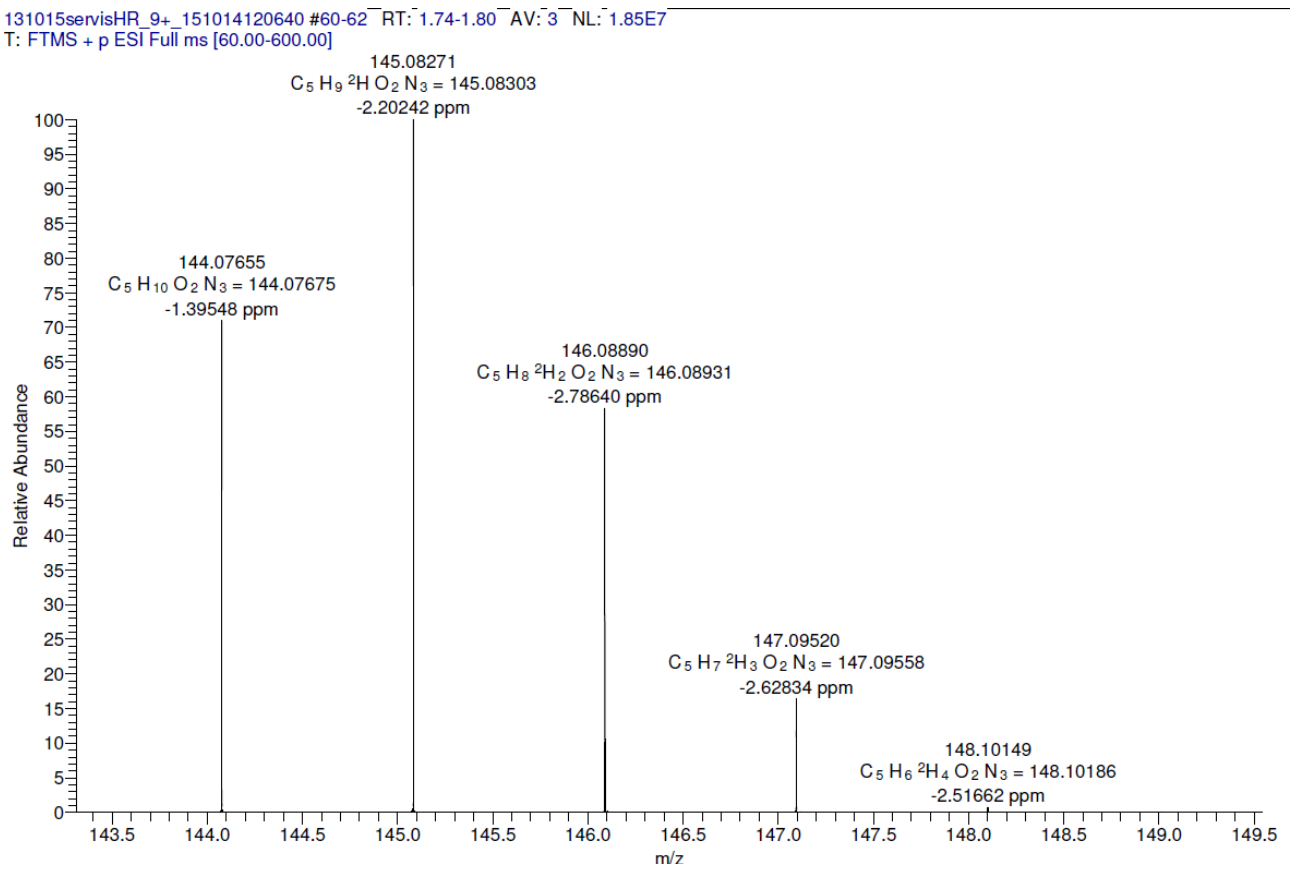

Figure S2. HRMS of [^{2}H]ATPCA drawing intensities of deuterium-multilabeled ligand

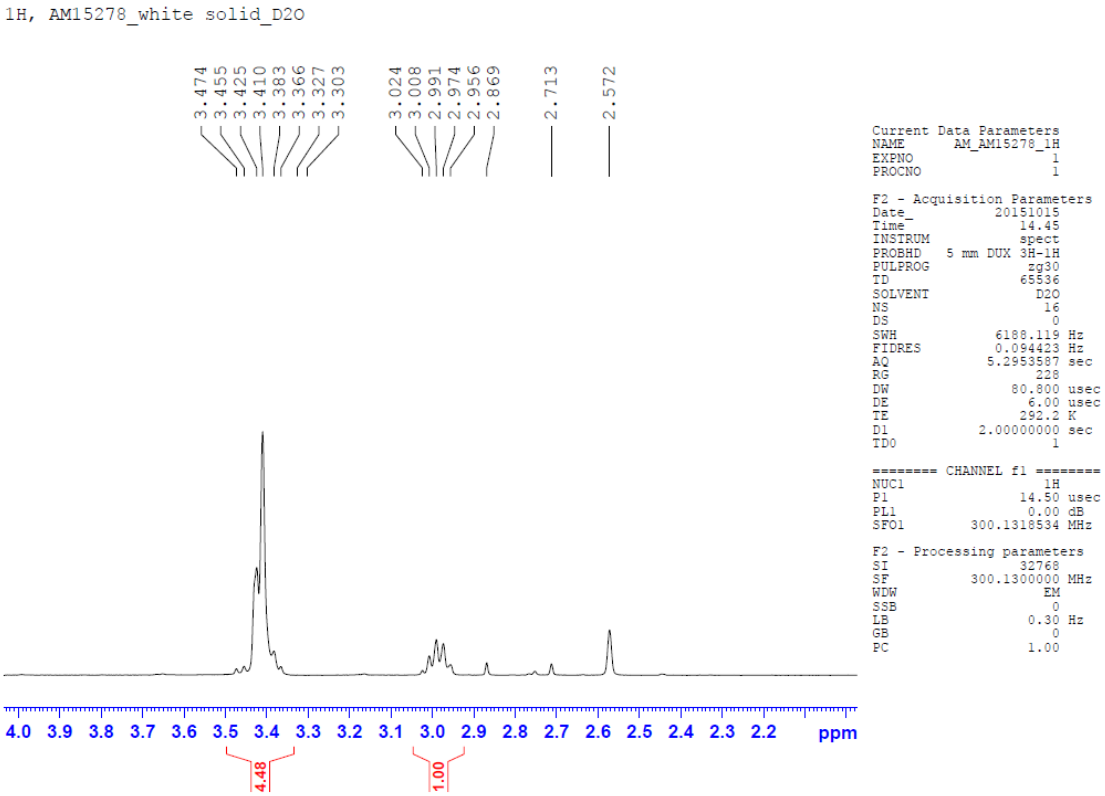

Figure S3. ^{1}H NMR (300 MHz) of [^{2}H]ATPCA (crude) in D_2O

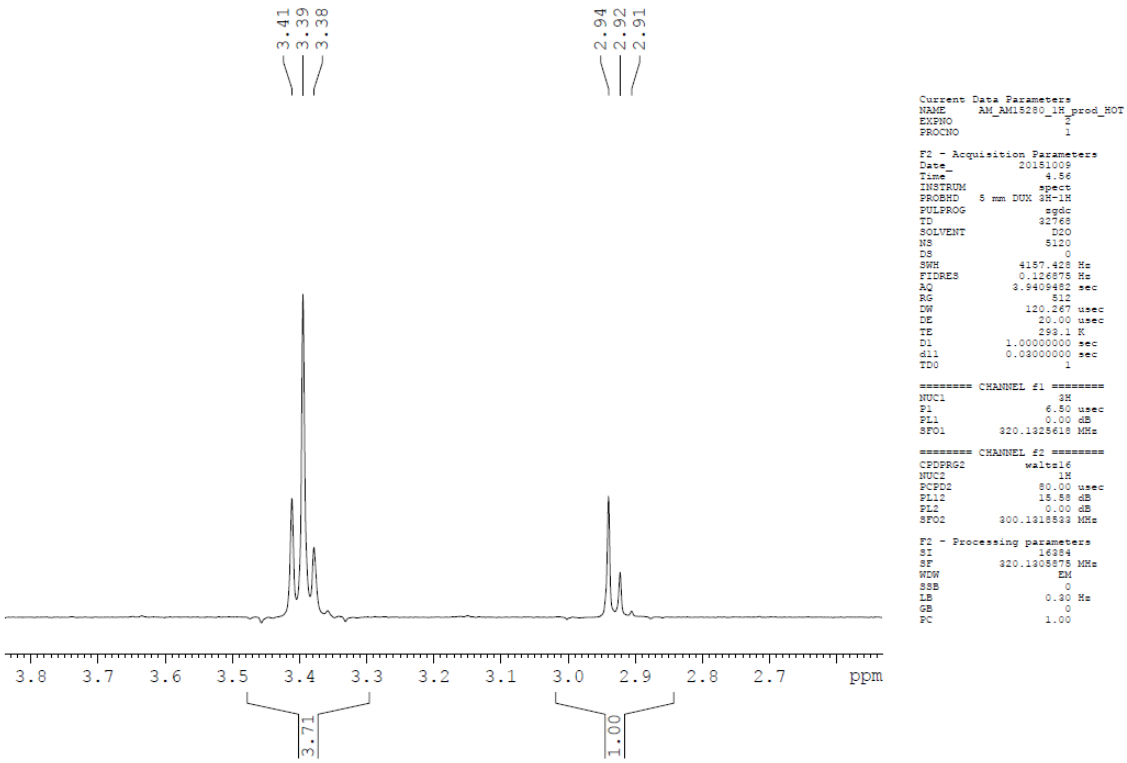

Figure S4. ^{3}H-^{1}H decoupled NMR (320 MHz) of [^{3}H]ATPCA (20 mCi) in D_2O

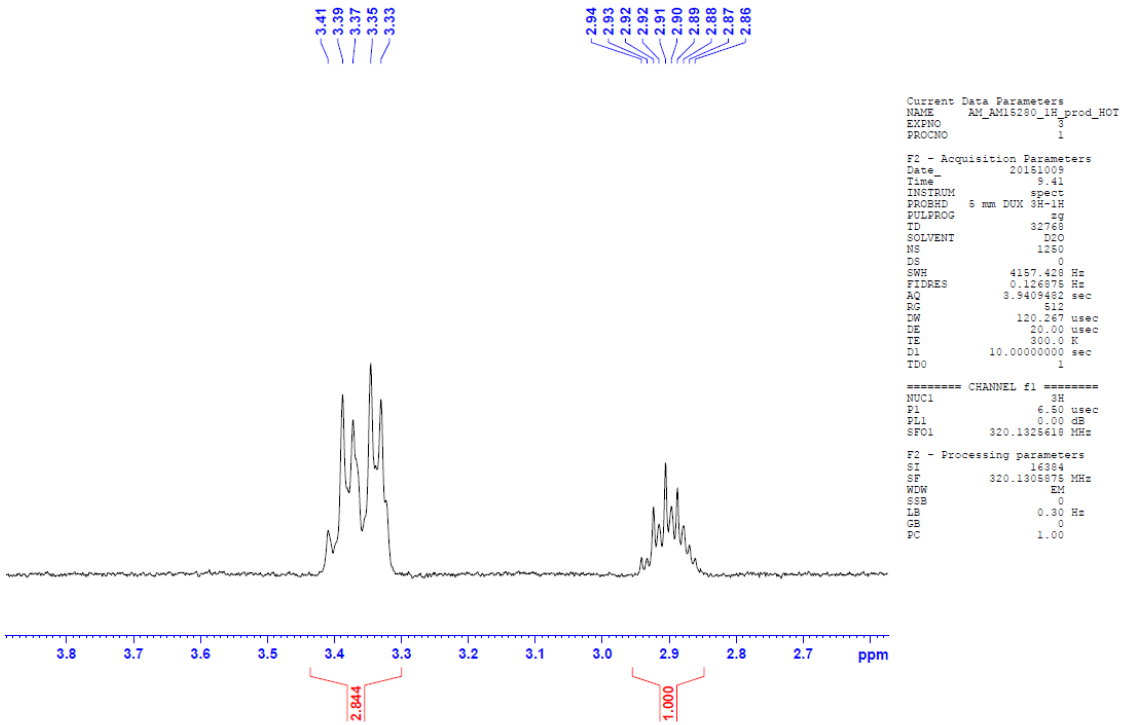

Figure S5. $^{3}H(^{1}H$ coupled) NMR (320 MHz) of [^{3}H]ATPCA (20 mCi) in D_2O

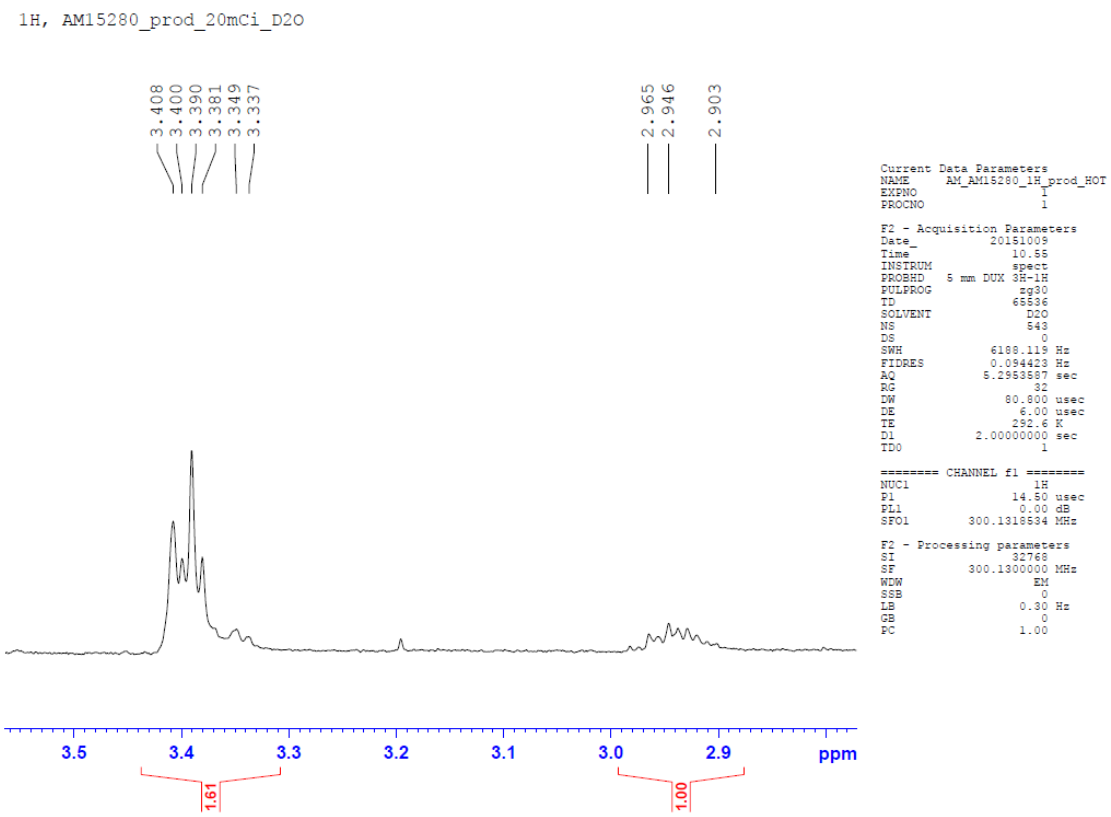

Figure S6. ^{1}H NMR (300 MHz) of [^{3}H]ATPCA (20 mCi) in D_2O