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A functional role for both GABA transporter-1 and GABA transporter-3 in the modulation of extracellular GABA and GABAergic tonic conductances in the rat hippocampus

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SUPPLEMENTAL MATERIAL

1. Supplemental Methods

1A. Western blot studies

Rats were killed using an overdose of isoflurane (Merial Animal Health Ltd., Harlow, UK). Brains were removed and 1 mm-thick brains sections were obtained using a brain matrix (Alto; World Precision Instruments, Sarasota, FL, USA). Hippocampal subregions (CA1, CA3

and dentate gyrus) were microdissected on ice under a microscope (Leica MZ95) and flash frozen in liquid nitrogen and stored at -80°C until use.

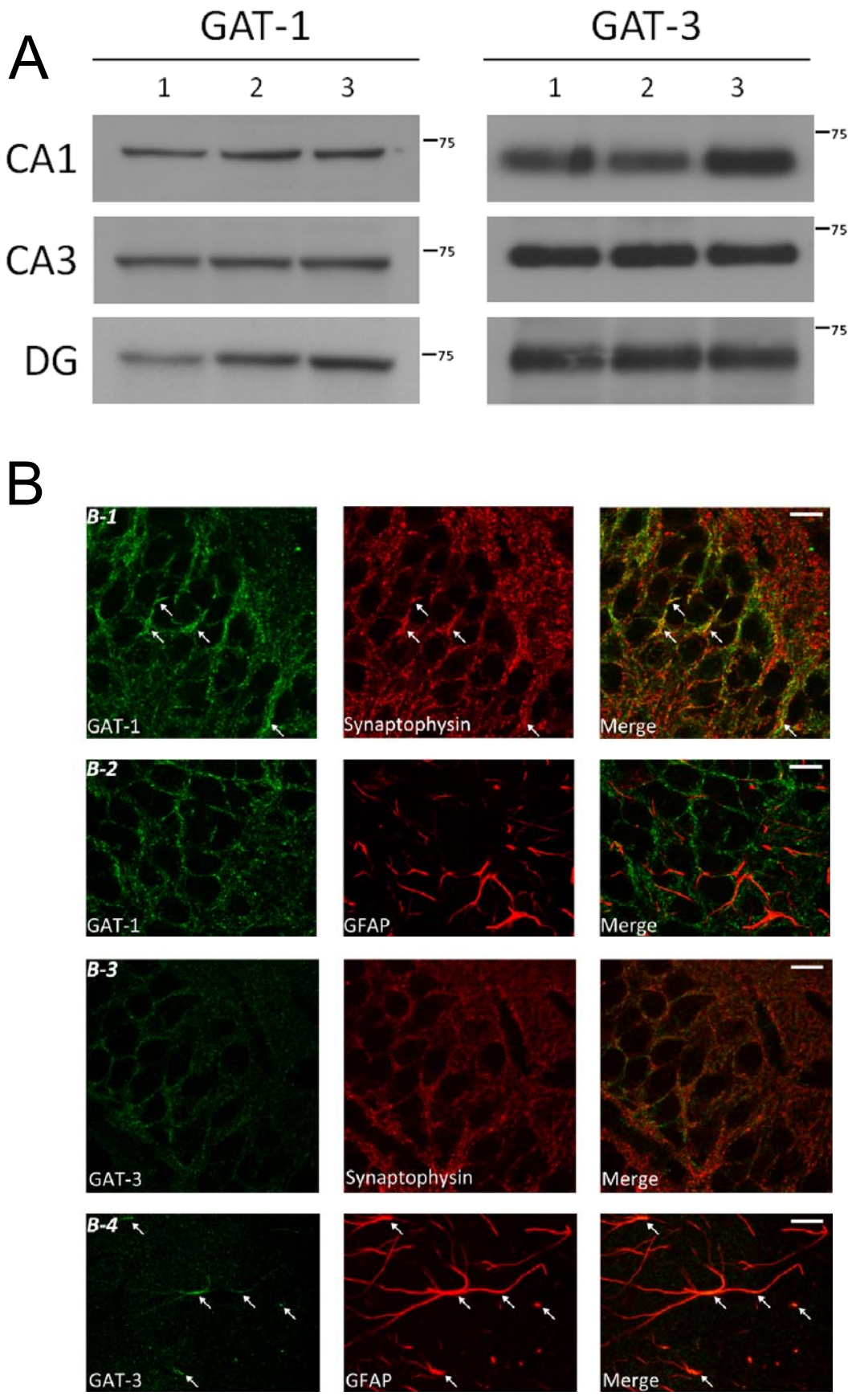
The tissues were homogenized in lysis buffer containing 50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and a protease inhibitor cocktail (Complete EDTA-free, Roche Applied Science, Burgess Hill, UK). The lysates were centrifuged at 1,000 x g for 15 minutes. The supernatants were kept and rotated for 1 hour at 4°C, again centrifuged at 20,000 x g for 15 minutes and diluted 10 times with lysis buffer. The diluted lysate was solubilized in Laemmli sample buffer, denaturated at 95°C and resolved on 10% polyacrylamide gel. Proteins in the gel were transferred to PVDF membranes (Bio-Rad, Hercules, CA) and western blotting was performed in PBS containing 0.2% Tween (PBS-T). Membranes were blocked for 1 h in PBS-T containing 5% non-fat dried milk and incubated overnight with primary antibody (rabbit anti-GAT-1, 1:5,000, ab426, Abcam (Cambridge, UK) or rabbit anti- GAT-3, 1:500, AB1574, Millipore) in PBS-T containing 1% BSA at 4°C with gentle agitation. After three washes, membranes were incubated for 1 hour at room temperature with HRP-peroxidase conjugated secondary antibody (goat anti-rabbit IgG, Jackson Immunoresearch Laboratories, West Grove, PA, USA). After six successive washes, immunoreactivity on the membranes was revealed using ECL or ECL Plus Western blotting detection kits (GE Healthcare Life Sciences, Little Chalfont, UK).

1B. Immunofluorescence studies

On the day of the experiment, rats were quickly anesthetized with isoflurane and received an overdose of pentobarbital (Euthatal, 200 mg/kg body weight i.p., Merial Animal Health Ltd.) after which they were transcardially perfused with ice-cold saline followed by 500 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and cut into 50 µm-thick horizontal sections using a vibratome (Leica VT 1000S, Leica, Nussloch, Germany) and stored at 4°C until use.

Free floating sections were incubated for 1 hour in blocking solution (2% BSA, 10% normal goat serum (NGS), 0.2 M glycine, and 0.1% Triton X-100 in Phosphate Buffered Saline (PBS)) and then incubated overnight with one of the following combinations of primary antibodies diluted in incubation solution (0.8 % BSA, 1% NGS and 0.1 % Triton X-100 in PBS): (a) rabbit anti-GAT-1 (1:100; AB1570, Millipore, Billerica, MA, USA) and mouse anti-GFAP (1:1,000; G3893, Sigma Aldrich, St. Louis, MO); (b) rabbit anti-GAT-1 and mouse anti-synaptophysin (1:1,000; Clone SY38, DAKO, Ely, UK); (c) rabbit anti-GAT-3 (1:50; AB1574, Millipore) and mouse anti-GFAP antibody; or (d) rabbit anti-GAT-3 and mouse anti-synaptophysin. Subsequently, sections were incubated for 2 hours with the secondary antibodies AlexaFluor®488 anti-rabbit IgG and AlexaFluor®546 anti-mouse IgG (Invitrogen, Grand Island, NY, USA) diluted 1:300 in phosphate buffer. Sections were carefully rinsed with phosphate buffer between each step. All incubations were carried out at room temperature and in darkness when using the fluorescent antibodies. Sections were mounted onto poly-L-lysinated slides and cover slipped with Vectorshield anti-fading mounting media (Vector Laboratories Ltd., Burlingame, CA, USA). Sections were analyzed and images were taken using a Leica SP5-AOBS confocal laser scanning microscope attached to a Leica DM I6000 inverted epifluorescence microscope.

2. Figure S1

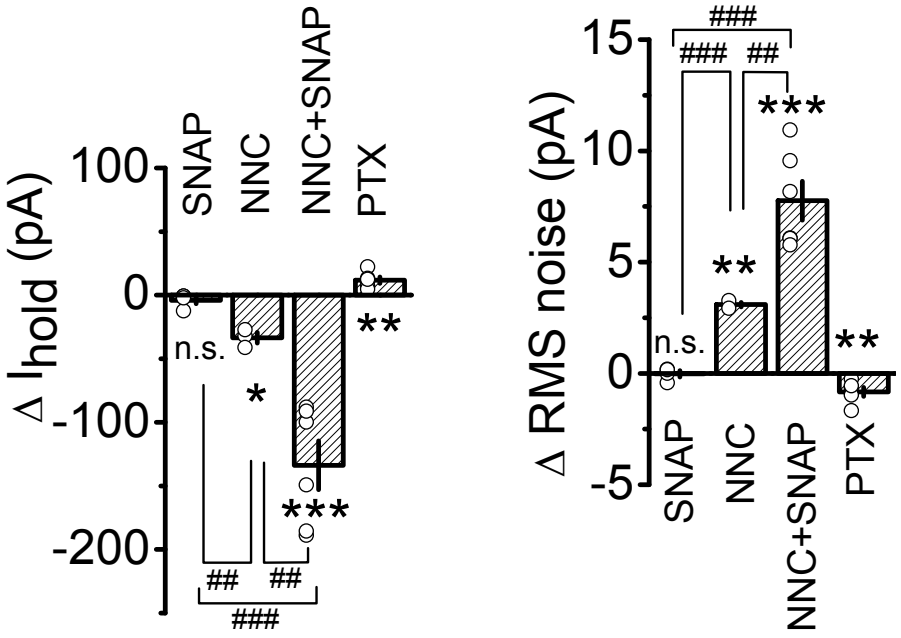

A
GAT-1
GAT-3
1
2
3
1
2
3
CA1
CA3
DG
75
75
75
75
75
75
B
B-1
GAT-1
Synaptophysin
Merge
B-2
GAT-1
GFAP
Merge
B-3
GAT-3
Synaptophysin
Merge
B-4
GAT-3
GFAP
Merge

3. Legend to Figure S1

A) Representative western blots showing the expression of GAT-1 (left panel) and GAT-3 (right panel) within the CA1, CA3 and dentate gyrus (DG) of the hippocampus in 3 individual rats (1, 2, 3).

B) GAT-1 and GAT-3 are differentially localized in the granular cell layer of the rat dentate gyrus as shown by dual-labeling immunofluorescence. Confocal Z-stack images representing the co-localization of GABA transporters (GAT-1: *B-1* and *B-2*, GAT-3: *B-3* and *B-4*; green fluorescence) with either synaptophysin (*B-1* and *B-3*; red fluorescence) or the astrocytic marker GFAP (*B-2* and *B-4*; red fluorescence). Co-localization of immunoreactive molecules is indicated by a yellow color in the merged images and examples are indicated by white arrows. GAT-1 showed clear co-localization with synaptophysin (*B-1*) but not with GFAP (*B-2*). In contrast, expression of GAT-3 seems to be restricted to astrocytes (*B-4*). Pixel sizes are 90.9, 85.7, 84.5 and 105.7 nm, for figures *B-1*, *B-2*, *B-3* and *B-4*, respectively. Scale bar = 10 μm.

4. Figure S2



5. Legend to Figure S2

Summary graphs showing changes in holding currents (left panel) and RMS noise (right panel) in the absence of TTX (see Methods in manuscript) following application of SNAP-5114 (n=4), NNC-711 (n=3), in the presence of both drugs (n=6), and after a blockade of $GABA_A$ receptors by picrotoxin (n=6). One way ANOVA holding current $F_{3,15} = 42.39$, $p < 0.001$; one way ANOVA RMS noise $F_{3,15} = 52.59$, $p < 0.001$. n.s., non significant; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, compared to the baseline condition (paired t-test); ## $p < 0.01$ and ### $p < 0.001$ (unpaired t-test). ΔI_{hold} and ΔRMS noise values represent changes from the baseline condition.