Homeostatic Regulation of NCAM-polysialylation is Critical for Correct Synaptic Targeting

Abbreviated Title: PSA Regulates Axonal Targeting

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Supplemental Material and Methods

Measurement of PSA and pPSA-expression

Slice cultures were solubilized at 4° C for 1h in buffer containing 150 mM NaCl, 10 mM Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 % Triton and a protease inhibitor cocktail P 2714 (Sigma), diluted 1:250 at pH 7,4. Solubilisates were centrifuged at 30,000 g for 15 min and supernatants were collected and further analyzed by western blot analysis. Samples were separated on SDS-polyacrylamide gels (BioRad) and transferred to nitrocellulose filters. The blots were blocked with 4 % fat-free dry milk powder in PBS, incubated with the respective primary antibodies or with streptavidin conjugated with peroxidase, washed with PBS and incubated with the appropriate secondary antibodies. After washing, the proteins were detected by enhanced chemiluminescence (Amersham Buchler) according to the manufacturer's instructions, and visualized by

exposing the blots to a Fuji imager system (LAS) for time periods between 10 and 120 sec. Monoclonal anti-PSA antibody was used as IgG [1]. Monoclonal antibody against pPSA (13D9), which contains the metabolized chemical engineered sialic acid precursor ManNProp was produced as described [2]. Antibodies to actin were purchased from Sigma and mouse immunoglobulins conjugated to HRP were obtained from Dianova.

Preparation of primary hippocampal neuronal cultures

Primary hippocampal neurons were prepared from embryonic day 18 rat Wistar pups as described [3]. Briefly, neurons were cultured at a density of 120.000 cells/well on poly-L-lysine coated glass cover slips in neurobasal A medium supplemented with 2% B27 (Invitrogen), 0,5 mM glutamine [4] and antibiotics (penicillin and streptomycin). One day after preparation neurons were treated with ManNProp for another 14 days. After 12 days in vitro (DIV12) neurons were transfected (Effectene, Quiagen) with an EGFP-encoding plasmid and fixed two days later. Confocal stacks of control and treated neurons were taken on a Leica SP5 confocal microscope equipped with an 63x HCX PL APO 1,4 NA oil immersion objective. All morphometric measurements were performed with Image J by investigators blind to the experimental conditions. Neurons from at least three independent experiments were analyzed up to a total dendritic length of 1000 µm per condition.

Electrophysiology

The DG-CA1 co-cultured slices were transferred to a submerged recording chamber where they were perfused at a rate of 2 ml/min with ACSF containing (in mM) 119 NaCl, 26 $NaHCO_3$, 10 glucose, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$ and 1 NaH_2PO_4 equilibrated with 95 % O_2 and 5 % CO_2. The recording chamber was mounted on an Olympus microscope equipped for IR-DIC microscopy. Whole-cell recording electrodes were filled with (in mM): 135 K-gluconate, 10 Hepes, 2 Mg-ATP, 20 KCl, 0.2 EGTA, pH was adjusted to 7.2 with KOH. All recordings were conducted at room temperature. CA1 pyramidal cells in these co-cultures had a resting membrane potential of -69 ± 3 mV (corrected for liquid junction potential) and an average input resistance of 344 ± 143 MΩ. To stimulate mossy fibers, low-resistance patch pipettes filled with external

solution were placed in the granule cell layer or the hilar region. EPSC amplitudes were stored and analyzed online and offline using Igor Pro (Wavemetrics Inc., Lake Oswego, OR, USA). Average values are expressed as mean ± standard error of the mean. Drugs used: group-II metabotropic glutamate receptor agonist DCG-IV (2 µM), GABAA-receptor antagonist GABAzine (1 µM), AMPA/kainate-receptor antagonist NBQX (10 µM).

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Supplemental Figures

Supplementary Figure 1

(a,b) To avoid bias which may arise by mistargeted NCAM in ManNProp-treated neurons, we have investigated the neuronal NCAM-distribution in neurons which were treated for 11 days in vitro (DIV) with 8 mM ManNProp. Overview (left, a) and higher magnification (right, b) confirms correct targeted NCAM on neuronal membranes. **(c)** Quantification of membrane-bound Neu5Ac and Neu5Prop in the brain of ManNProp-treated animals revealed strong Neu5Ac-expression and minimal Neu5Prop synthesis arguing against unspecific effects. However, as shown by others [5], in spite of the low metabolization rate of ManNProp, treatment with ManNProp was able to effectively decrease NCAM-polysialylation (see also **Fig.1; Fig. 5g** and **Suppl. Fig. 6e**). Remarkable, total amount of sialic acids remained stable during

treatment. **(d)** Analysis of sialic acids in the liver of treated animals revealed a lower total amount of membrane-bound sialic acids but a higher amount of metabolized ManNProp (detected as Neu5Prop). Scale bar: 25 µm

Supplementary Figure 2

(a,b) Mossy fibers (MF) treated with 8 mM of the natural sialic acid precursor ManNAc show a normal termination in the stratum lucidum of the CA3-region. Higher magnification of the biocytin traced granule cells in the dentate gyrus (DG) revealed granule cells with typical dendritic morphology. **(c)** To avoid bias by indirect effects i.e. caused by cell death in the CA3-region, we have assessed the survival of neurons in the CA3-region. As shown in **Suppl Fig. 2c**, the CA3-region contains CA3-pyramids with typical morphology. **(d)** Stereological analysis of pyknotic cells in the CA3-region revealed no difference between control slices (n = 5) and ManNAc (n = 5) and ManNProp-treated slices (n = 5), respectively. Scale bars: 100 µm (a) and 50 µm (b,c).

Supplementary Figure 3

Merged images of CB and pPSA-expression (detected with the 13D9-antibody) show double-labeled fibers (yellow) which aberrantly entered into the CA3-region. While almost all aberrant fibers were CB and pPSA-positive, CB-positive MF-boutons were devoid of pPSA. Scale bar: 50 µm.

Supplementary Figure 4

Schematic drawing of the co-cultured DG-CA1 hippocampal slices.

Supplementary Figure 5

(**a-c**) After PSA-inhibition the infrapyramidal MF bundle was significantly longer in hippocampi from ManNProp-treated animals, which is a phenotype characteristic for *ST8SiaII*$^{-/-}$ mice (Angata et al., 2004). This finding confirms the specificity of ManNProp treatment, which functionally inactivates the developmentally expressed ST8SiaII (see Fig 1b; [6]). (**d-f**) In 4 week-old ManNProp-treated animals, all

MF of the longer infrapyramidal tract expressed calbindin but only some expressed PSA. Notably, PSA was expressed at lower levels and was not found in the longer infrapyramidal MF (arrows). PSA-expression in a subset of MF may have resulted from compensatory effects via ST8SiaIV which may eventually have led to an unaltered PSA-expression in MF of adult animals as shown by others [7]. Scale bar 50 µm (a-f).

Supplementary Figure 6

Golgi-impregnation of hippocampal pyramidal neurons of ManNProp-treated animals

Overview (**a**) and higher magnification (**b,c**) of CA1 neurons close to the CA3 border. (**b-c**) Note that CA1 pyramidal neurons lack giant postsynaptic excrescences while their dendrites are covered with spines. (**d**) Examples of CA3 pyramidal neurons that bear typical thorny excrescences on the proximal portion of their apical dendrites. This confirms that ManNProp-treatment *per se* does not inhibit the formation of characteristic postsynaptic structures. Scale bar, 100 µm (a,b,d). 50 µm (c)

Suppl. Fig. 1

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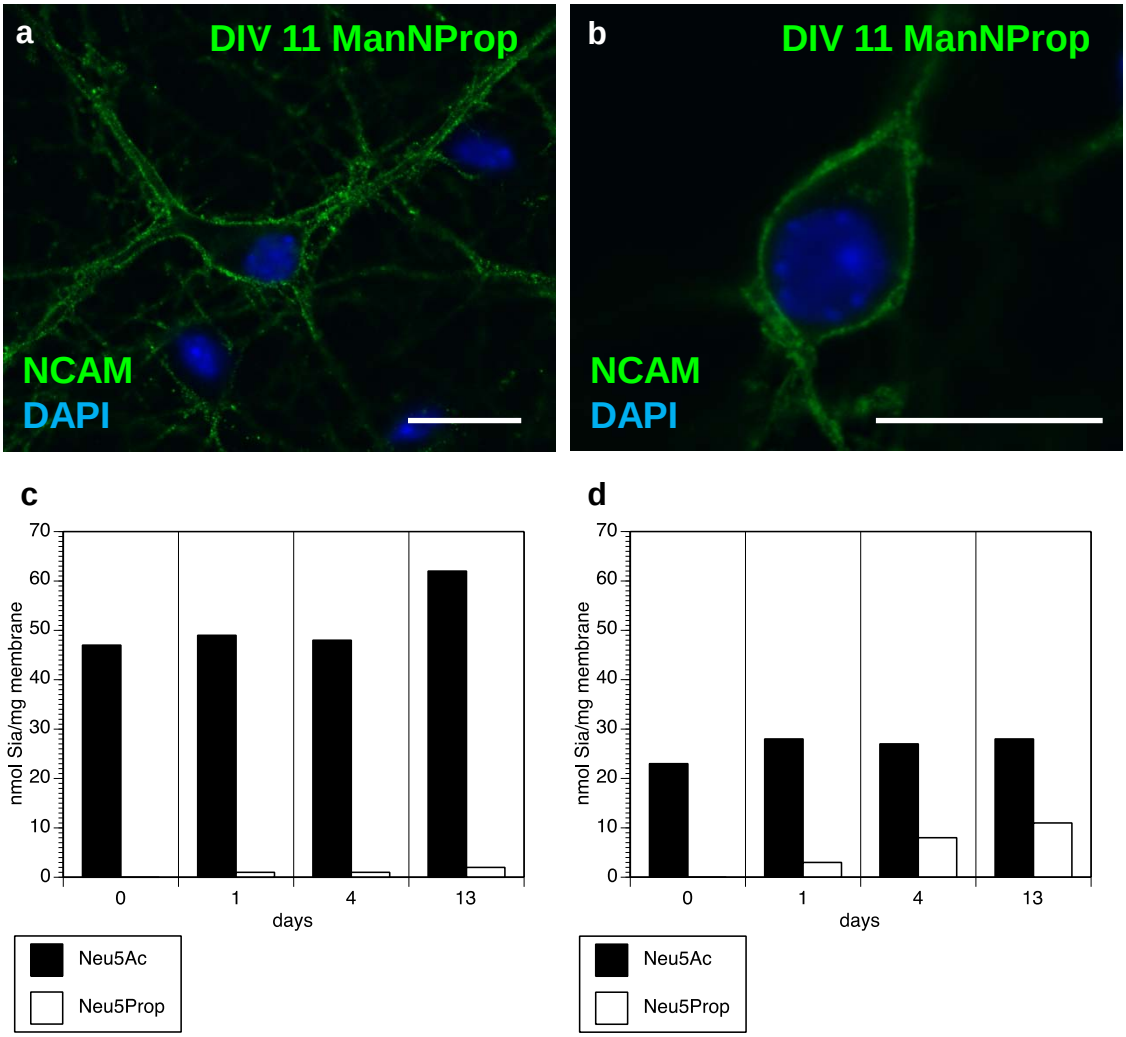

Suppl. Fig. 2

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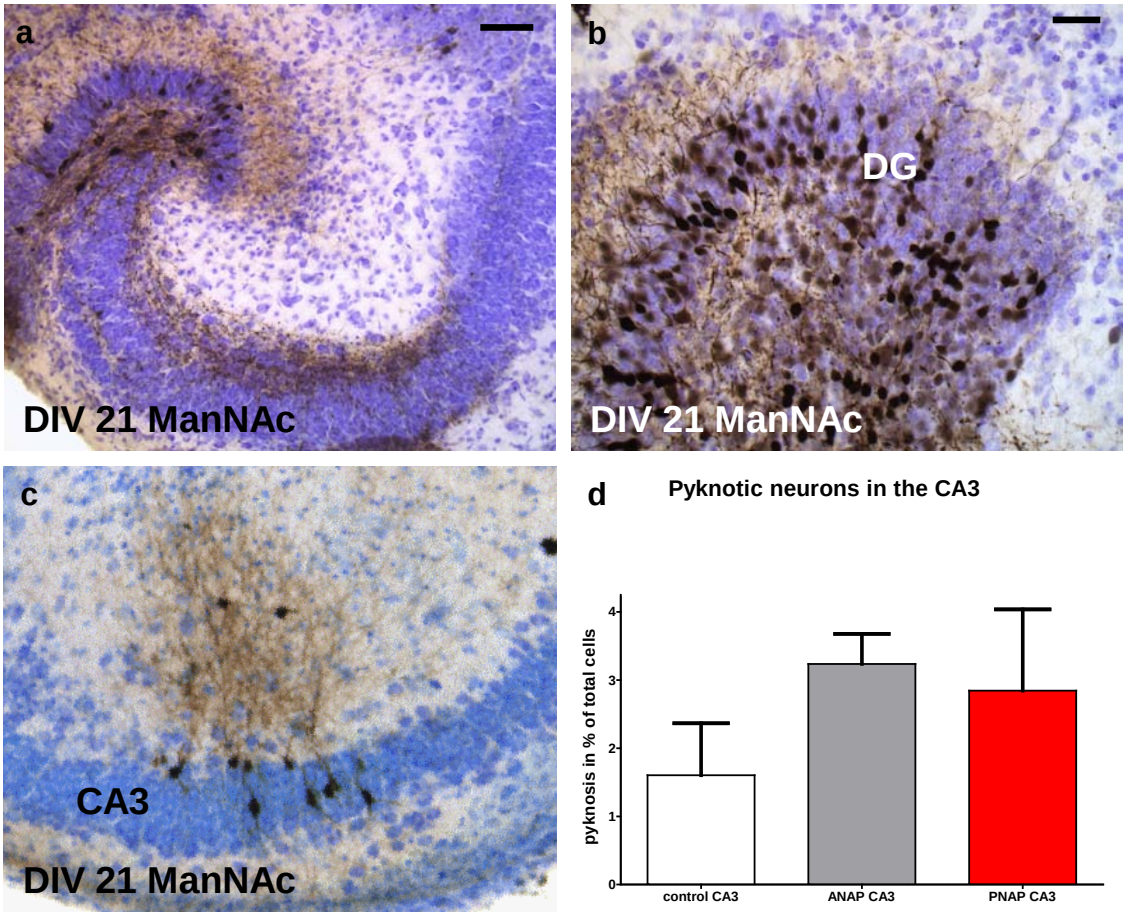
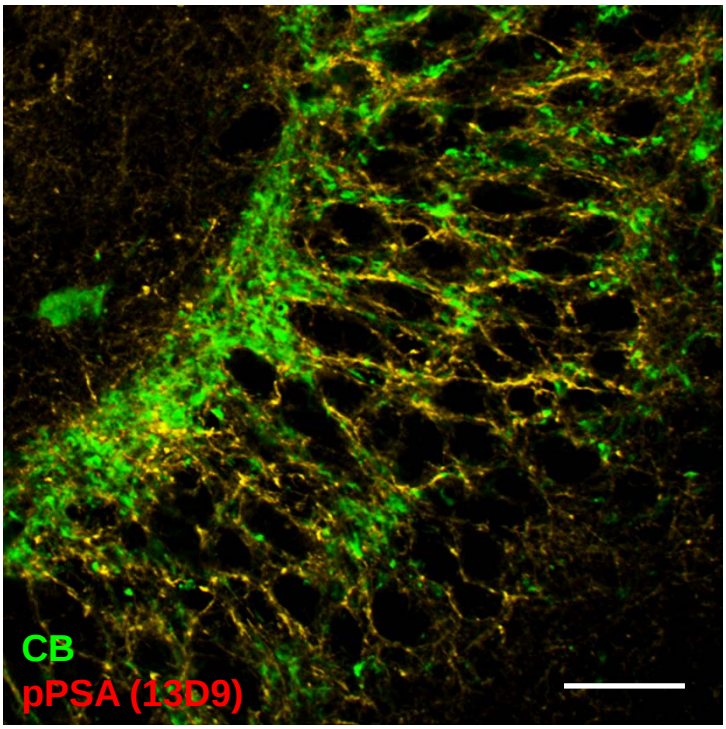


Suppl. Fig. 3 **Vogt et al.**



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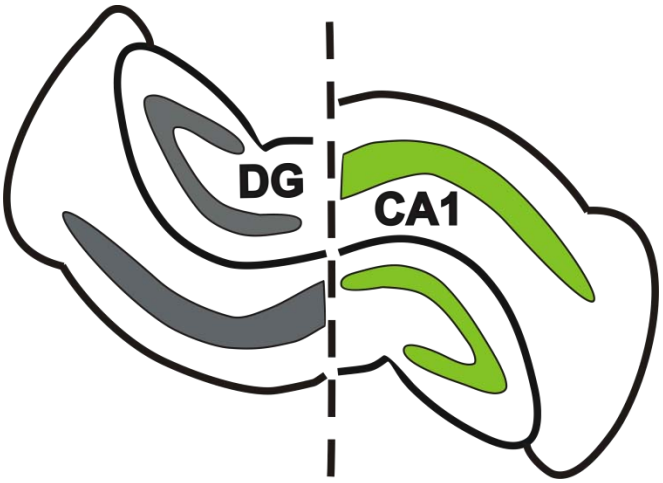

Suppl. Fig. 5

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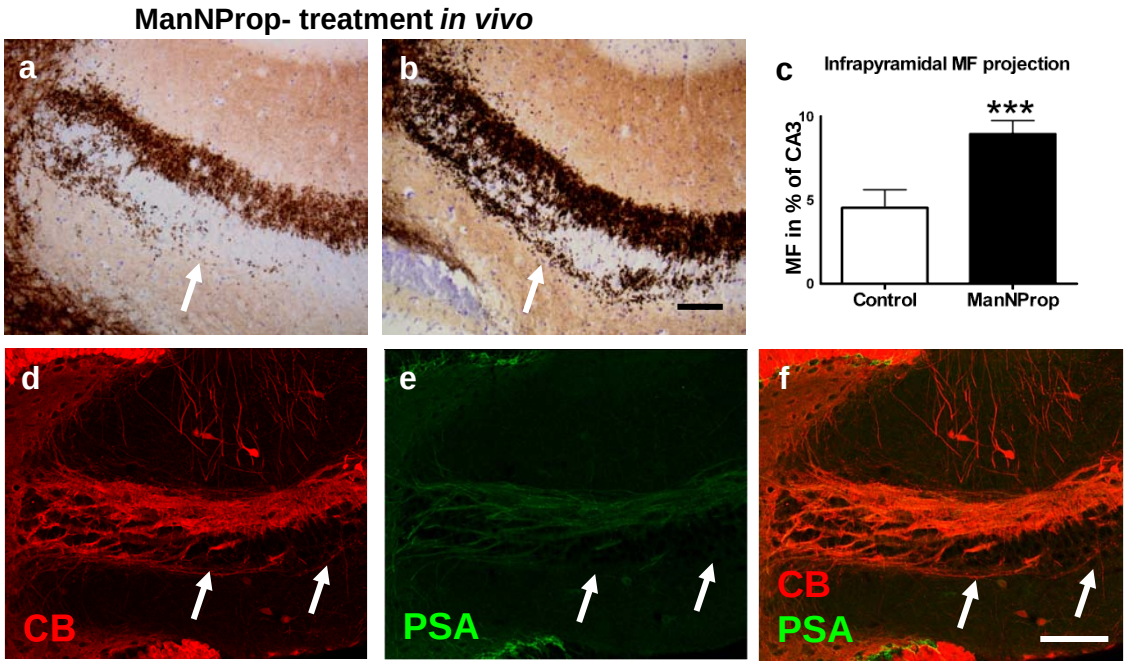

Suppl. Fig. 6

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