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Interfering polysialyltransferase ST8SiaII/STX mRNA inhibits neurite growth during early hippocampal development

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Abstract **Polysialic acid (PSA) attached to NCAM is involved in cell–cell interactions participating in structural and functional plasticity of neuronal circuits. Two polysialyltransferases, ST8SiaII/STX and ST8SiaIV/PST, polysialylate NCAM. We previously suggested that ST8SiaII/STX is the key enzyme for polysialylation in hippocampus. Here, polysialyltransferase mRNA interference experiments showed that, knock down of ST8SiaIV/PST transcripts did not affect PSA expression, but PSA was almost absent from neuronal surfaces when ST8SiaII/STX mRNA was interfered. Non-polysialylated neurons bore a similar number of neurites per cell than polysialylated neurons. However, non-polysialylated processes were shorter and a lower density of synaptophysin clusters accompanied this reduced neuritic growth. Therefore, ST8SiaII/STX expression is essential to allow a correct neuritic development at initial stages of hippocampus ontogeny.**

Keywords: Polysialic acid; Synaptogenesis; RNAi; Neuritic length; Non-polysialylated neurons

1. Introduction

Polysialic acid (PSA) is a post-translational modification consisting of an homopolymer of alpha (2–8)-linked sialic acids [1] that participates in neural development. Neural cell adhesion molecule (NCAM) is the major PSA carrier. Highly polysialylated NCAM (PSA-NCAM) expression is developmentally regulated. During neonatal development, PSA-NCAM levels decrease, and, in adult nervous system, they become restricted to regions of morphological and/or physiological plasticity, such as hippocampus [2,3]. Additionally, PSA expression has been observed in many malignant tumors, such as neuroblastoma and non-small-cell lung cancer [4]; where PSA may be involved in high mitosis ratio and metastasis.

PSA is synthesized by two polysialyltransferases, ST8SiaII/STX [5,6] and ST8SiaIV/PST [7,8], which are 56% identical in amino acid sequence. Polysialyltransferase mRNA distribution has been observed throughout the vertebrate central nervous system, although they are expressed with different temporal and spatial patterns [9]. Both polysialyltransferase mRNAs levels parallel PSA-NCAM expression. ST8SiaII/STX-deficient-mice resulted in an alteration of axonal targeting involving hippocampus infrapyramidal mossy fibers in association with ectopic synapses where these fibers abnormally terminate at CA3a [10]. On the contrary, mice homozygous for the null mutation of ST8SiaIV/PST gene showed only subtle alterations in hippocampal long term potentiation in CA1 area during adulthood [11]. In our laboratory, we quantified polysialyltransferase mRNA levels during hippocampus development. We observed a strong down-regulation in ST8SiaII/STX mRNA expression. In contrast, ST8SiaIV/PST mRNA showed low levels that were not regulated. In addition, ST8SiaII/STX overexpression in primary hippocampal cultures induced neuronal death independently of enzyme activity [12]. These results led us to propose that ST8SiaII/STX is the responsible enzyme for polysialylation during hippocampus ontogeny. However, how an altered PSA formation modulates neuronal development at the cellular level remains elusive.

To gain insight, we depleted individual hippocampal neurons in culture of ST8SiaII/STX mRNA by RNA interference (RNAi). Neurons with ST8SiaII/STX mRNA interfered lacked PSA on their membranes. Moreover, these non-polysialylated cells showed a reduced neuritic growth and a lower number of synaptophysin clusters. Our results indicate that ST8SiaII/STX is involved in early neurite development.

2. Materials and methods

2.1. Animals

Animals used were Sprague Dawley rats maintained at Facultad de Ciencias Veterinarias (Buenos Aires, Argentina). All animal procedures carried out in this study were in accordance with the guidelines laid down by the NIH regarding the care and use of animals for research.

2.2. Cell culture

Hippocampal primary cultures were established from 19-day-old fetal Sprague Dawley rat hippocampi as described previously [12]. Tissue was treated with 0.25% trypsin in Hank's solution for 15 min at 37 °C. A single-cell solution was prepared by dissociation with a narrow polished Pasteur pipette in 10% horse serum supplemented MEM 1× (MEM containing 4.5 g/l glucose, 2 mM glutamine, 10 μM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin). Cells were seeded on coverslips coated with 0.1 mg/ml poly-L–lysine hydrobromide (Sigma, St. Louis, MO) and 20 μg/μl laminin (Gibco, Carlsbad, CA) at a density of 30,000 cells/cm^2. After 2 h, medium was changed to MEM/N2 (MEM 1× with 1 g/l ovalbumin and B27 serum-free supplements from Gibco).

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Abbreviation: PSA, polysialic acid

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2.3. RNA interference

Interference vectors were obtained by cloning hairpin siRNA inserts into pSilencer (Ambion Inc., Austin, TX) following manufacturer's instructions. To design hairpin siRNA template oligonucleotides, we used the available tools at www.ambion.com. ST8SaII/STX and ST8SiaII/PST siRNA targets chosen were 5′GCCTGGAGACATTATTCATT3′, nucleotides 354–373 of rat ST8SiaII/STX (GenBank Accession No. # L13445) and 5′TGCACTATAAGTCTACTCCTT3′, nucleotides 31–49 of rat ST8SiaII/PST (GenBank Accession No. # U90215). To identify interfered cells, green fluorescent protein (GFP) gene was cloned in pSilencer vector. We made controls to evaluate transfection protocol and GFP expression effects. There were no differences in the parameters measured between GPF transfected and non-transfected cells. Therefore, GFP positive cells were used as control to evaluate RNAi effects. In addition, as control, we performed assays with STX or PSA pSilencer vector without GFP where transfected cells were identified by PSA expression. These experiments, without GPF, gave similar values for neurite length measurements to those observed with pSilencer vectors including GFP.

After 1 day in culture, cells were transfected with interference vectors. Transfections were performed with 3 μg Qiagen-purified DNA (Qiagen GmbH, Germany) accomplished with 1 μl Lipofectamine 2000 (Gibco) per well (in a 24 wells/plate format) in MEM/N2. Then cells were incubated with transfection mix for 4–6 h at 37 °C. Finally, media was changed to fresh MEM/N2. After 3 days, cells were fixed to evaluate PSA expression on GFP expressing cells. Transfection efficiency was about 10–20 % for all vectors assayed.

2.4. Immunocytochemistry

Neuronal cells were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS), blocked with 3% (w/v) bovine serum albumin and then, treated with primary monoclonal antibodies 12E3 anti-PSA (1:1000, kindly provided by Dr. Seki (Japan, [13])); anti-tubulin (1:2000; Clone B-5-1, Sigma) and anti-synaptophysin (1:400, Synaptic Systems GmbH, Germany). Next, cells were treated with the appropriate secondary conjugated antibodies. Finally, cells were washed and then, mounted with FluorSave reagent (Calbiochem, San Diego, CA) as anti-fading reagent. Fluorescent images were obtained using a Nikon E600 microscope equipped with epifluorescence illumination (Nikon, Japan) with 40× and 100× oil-immersion lens.

2.5. Morphometric analysis and synaptophysin expression

To evaluate neurite length, cells transfected with STX-pSilencer and fixed three days later, were immunolabeled with anti-PSA and anti-tubulin antibodies as described above. Individual processes were traced manually and measured with ImageJ, a java-based image analysis program developed at the US National Institutes of Health and available on the internet [14]. To carry a blind-assay neurite length measurements were evaluated on tubulin channel only and then lengths were matched to PSA positive or negative cells. There was at least one PSA positive and negative cell per picture analyzed. PSA-negative cells were compared to PSA-positive cells within the same culture. Also, neurite number per cell was analyzed. Axons and dendrites were distinguished for their morphometric characteristics (length, calibre, etc.). Values are expressed as mean ± standard error. Statistical differences were evaluated with independent parametric *t*-test.

Synaptophysin cluster density was analyzed 8 days after RNAi. Cells were immunolabeled with anti-PSA and anti-synaptophysin antibodies as described. Number of immunopositive synaptophysin puncta was counted in 20 μm-dendrite fragments measured from the neuronal body from 55–60 different neurons per group from two independent experiments. Group means were then analyzed for overall statistical significance using the non-parametric Mann Whitney *U*-test, 2-tailed.

All statistics calculations were performed with the software Analyse-it® for Microsoft Excel.

3. Results

3.1. ST8SIAII/STX mRNA interference

We used RNAi to knock down endogenous polysialyltransferase gene expression in hippocampal neurons developing in culture. The targeting siRNA sequences for each polysialyltransferase have no significant homology with other known genes, including the other polysialyltransferase. ST8SiaII/STX mRNA expresses at higher levels at initial culture stages [12]; thus, RNAi vectors were transfected in 1-day cultures. GFP subcloned in RNAi vector identified transfected cells. After 3 days in culture, we analyzed PSA expression on GFP positive cells. Cells transfected with PST pSilencer-GFP expressed PSA, as revealed by antibody staining (Fig. 1A, arrowheads). More than 80% of PST pSilencer transfected neurons expressed PSA. In contrast, STX pSilencer-GFP-expressing cells (Fig. 1A, arrows) did not express PSA. Only about 10% of STX pSilencer transfected cells expressed PSA (Fig. 1B), indicating that interfering ST8SiaII/STX gene reduced polysialylation.

3.2. Non-polysialylated neurons develop shorter neurites

Non-polysialylated cells survived and developed processes without obvious alterations through 9–10 days in culture. We compared neurite outgrowth from polysialylated and non-polysialylated cells. ST8SIaII/STX mRNA was interfered at day 1 after plating and cultures were fixed and immunostained with anti-tubulin and anti-PSA antibodies 3 days later (Fig. 2A). Both experimental groups bore a similar number of primary neurites per cell (about 5 neurites per cell, Fig. 2B). Process length measurements showed that non-PSA-expressing cells extended significantly shorter processes than control polysialylated cells (98.6 ± 12.2 vs. 184.4 ± 30.6 μm;

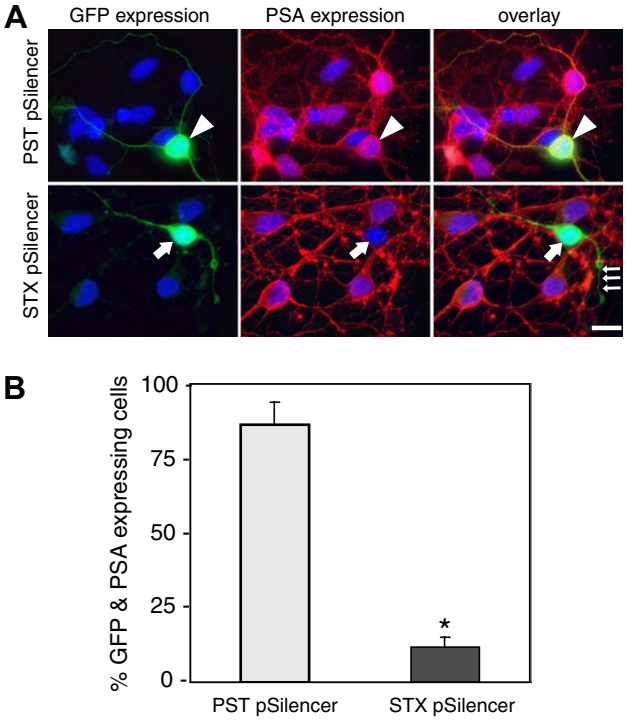

Fig. 1. ST8SiaII/STX mRNA interference decreases PSA expression from hippocampal neurons in culture. Hippocampal primary cell cultured for 1 day were transfected with STX and PST pSilencer-GFP constructs, fixed 3 days later and immunolabeled with anti-PSA antibody. (A) GFP expression and PSA labeling are shown. Arrows show a STX pSilencer GFP-expressing cell in which PSA expression is absent. On overlay picture, little arrows show a process from this cell. PST pSilencer GFP transfected cells do express PSA (arrowheads). Nuclei were labeled with DAPI staining. Bar, 20 μm. (B) Percentage of transfected cells expressing PSA (about 100 cells from two independent experiments were counted), $p < 0.005$, Mann–Whitney *U*-test, 2-tailed.

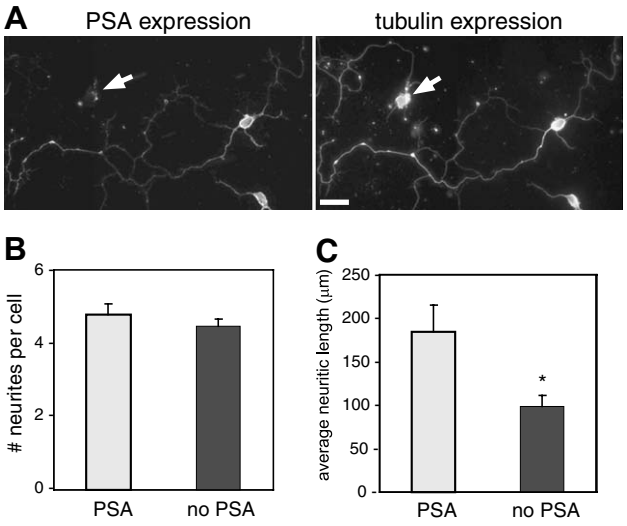

Fig. 2. Non-polysialylated neurons develop shorter neurites. ST8SiaII/STX mRNA was interfered in 1 day-primary hippocampus cultures. Cells were fixed after 4 days in vitro and treated with anti-PSA and anti-tubulin antibodies (A). The number of neurites per cell (B) and neurite lengths (C) were evaluated with ImageJ software (see Section 2 for details). Non-polysialylated cells (arrow) developed a similar number of neurite per cell than non-interfered cells, but the processes were near 50% shorter. Data given are mean values from three independent experiments ± standard error. About 100 neurites were evaluated per experiment. Statistical differences were analyzed using the non-parametric Mann–Whitney *U*-test, 2-tailed. Asterisk indicates a statistically significant difference ($p < 0.005$). Bar, 50 μm.

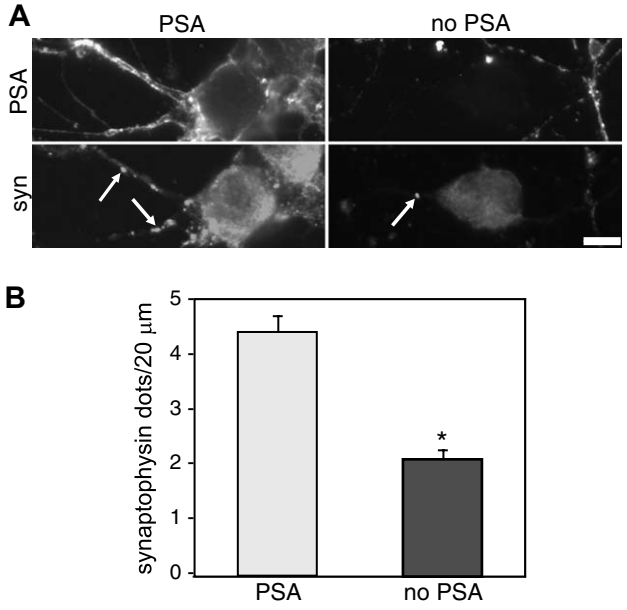

Fig. 3. ST8SiaII/STX mRNA interference reduces the number of synaptophysin immunoreactive clusters. ST8SiaII/STX mRNA was interfered in 1-day primary hippocampus cultures. Cells were fixed after 9 days in vitro and treated with anti-PSA and anti-synaptophysin antibodies (A). Number of immunopositive dots (arrows), probably representing clusters of synapses, was counted in 20 μm-fragment of dendrites measured from the neuronal body. Mean ± S.E.M. for 40–50 cells per group from two independent experiments are shown in B. Asterisk indicates a statistically significant difference ($p < 0.001$, Mann–Whitney *U*-test, 2-tailed). Scale bar = 10 μm.

Fig. 2C) . Distinction between axons and dendrites extension resulted in a similar difference to that showed for average neurite lengths (not shown). These results indicate that neurons without PSA on their surfaces (i.e. after ST8SiaII/STX gene interference) sprout neurites at the same ratio than control PSA expressing neurons. However, after 4 days in culture, non-polysialylated cells failed to extend their process, irrespective of its nature (axon or dendrite).

3.3. ST8SiaII/STX RNAi reduces the number of synaptophysin clusters

Next, we analyzed whether ST8SiaII/STX mRNA interference could also affect synaptogenesis. Synapse formation for dissociated neurons in culture strongly correlates with the acquisition of focal accumulations of labeling for synaptic vesicle proteins along cell bodies and dendrites [15]. We examined the distribution of synapses by immunolabeling for synaptophysin, a synaptic vesicle protein that reliably indicates the location of synapses [16,17]. For these experiments, cells were transfected with RNAi vector at 1 day in vitro and fixed 8 days post transfection and then, immunolabeled with anti-synaptophysin and anti-PSA antibodies. Synaptophysin clusters were quantified per 20 μm-dendritic fragments from PSA positive and negative cells. Non-PSA expressing cells exhibited lower synaptophysin puncta density than PSA expressing cells (Fig. 3A). PSA deficiency reduced a 50% of synaptic dots compared with control cells (Fig. 3B). Altogether, these data indicate that PSA expression is necessary for neurite growth and for synapse establishment during early hippocampal neuron development.

4. Discussion

Previously, we proposed that ST8SiaII/STX is the responsible enzyme for polysialylation during hippocampus ontogeny [12]. In the present work, we looked for a role for ST8SiaII/STX at the cellular level. RNAi experiments pointed out that PSA expression is required for neurite extension because lack of PSA expression limited neurite growth and reduced synaptogenesis.

To evaluate, ST8SiaII/STX mRNA depletion in fetal hippocampal neurons developing in culture, we analyzed PSA expression. As PSA is the product of polysialyltransferase –ST8SiaII/STX and ST8SiaIV/PST – catalytic activity, variations in PSA expression may reflect polysialyltransferase activity. In fact, we found that ST8SiaIV/PST mRNA interference practically did not affect PSA expression. But, most STX pSilencer-expressing neurons did not express PSA. These results indicate that only ST8SiaII/STX loss-of-function abolished PSA expression, thus showing that ST8SiaII/STX is the enzyme responsible for polysialylation during early hippocampus development. In agreement with these results, Angata and colleagues (2004) described a lower number of PSA-expressing progenitor cells in the embryonic dentate gyrus from ST8SiaII/STX-deficient-mice [10]. Lack of PSA expression suggests that the other polysialyltransferase (ST8SiaIV/PST) does not counteract for ST8SiaII/STX absence. Thus, polysialyltransferase activities are not overlapped during hippocampus development confirming the genetic partitioning of PSA formation proposed by Angata et al. (2004), who suggested different roles for both polysialyltransferases, at least in hippocampus. Moreover,

ST8SiaIV/PST deficiency only affects synaptic plasticity in adult mice [11], confirming its involvement in neurological process distinct from those assigned to ST8SiaII/STX.

ST8SiaII/STX mRNA interference generated non-polysialylated neurons. These cells exhibited failures in neurite growth. This might be attributed to PSA absence itself or to failings in other adhesion molecule interactions on neuronal membranes. Although, neurons without PSA bore a proper number of processes, they were not able to extend them at the same ratio than polysialylated cells. Many previous studies have shown that NCAM interactions promote neuritic growth in different models [18,19]. However, PSA genetic ablation revealed that the major function of PSA is to mask NCAM to ensure organized NCAM contacts in time- and site-specific manner [20]. Therefore, ST8SiaII/STX mRNA interference and, consequently, PSA absence allows premature NCAM interactions resulting in shorter neurites. In the context of hippocampus development, it means that axons from ST8SiaII/STX-deficient neurons might not be able to reach their target. Histological data derived from ST8SiaII/STX-deficient-mice revealed that, even though hippocampal neurons express PSA – likely with a sugar chain quality different from wild type mice – their axons became mistargeted [10]. Therefore, both results indicate that failures in PSA expression as a consequence of ST8SiaII/STX knock down inhibit axon guidance during hippocampus ontogeny.

Our study shows that PSA and ST8SiaII/STX expression is related to synaptogenesis. Non-polysialylated neurons exhibited reduced synaptophysin immunoreactivity and lower synaptic cluster density. Moreover, ST8SiaII/STX overexpression affected cell survival in a temporal window linked to synaptogenesis onset (unpublished results). In addition, Dityatev et al. (2004) showed that enzymatic removal of PSA from hippocampal cells in culture also blocked synapse formation [21]. Both results indicate that PSA effect on synapse formation is independent from NCAM. On the contrary, injections of endo N – an enzyme that degrades the α (2–8)-linked polysialic acid chains – in brain mice resulted in the formation of ectopic mossy fiber synaptic boutons [22] and polysialylated mistargeted mossy fibers from mice lacking ST8SiaII/STX also form ectopic synapses [10]. However, those studies analyzed well-established synapses in adult animals and the present work provide data from early synaptogenesis onset. Then, PSA absence limits neurite outgrowth and prevents synapse establishment. In conclusion, ST8SiaII/STX expression during early hippocampus development should be accurately controlled to allow processes to reach their target and neurons become connected.

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