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Inhibition of brain ST8SiaIII sialyltransferase leads to impairment of procedural memory in mice

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ABSTRACT

Several glycoproteins in mammalian brains contain α2,8-linked disialic acid residues. We previously showed a constant expression of disialic acid (DiSia) in the hippocampus, olfactory bulb and cortex, and a gradual decrease of expression in the cerebellum from neonatal to senile mice. Previous publications indicate that neurite extension of neuroblastoma-derived Neuro2A cells is inhibited in the presence of DiSia antibody. Based on this, we treated Neuro2A cell cultures with RNA interference for ST8SiaIII mRNA, the enzyme responsible for DiSia formation. We observed that neurite extension was inhibited by this treatment.

Taking this evidence into consideration and the relationship of the cerebellum with learning and memory, we studied the role of DiSia expression in a learning task.

Through delivery of pST8SiaIII into the brains of C57BL/6 neonatal mice, we inhibited the expression of ST8SiaIII. ST8SiaIII mRNA and protein expressions were analyzed by real-time PCR and western blot, respectively.

In this work, we showed that pST8SiaIII-treated mice presented a significantly reduced level of ST8SiaIII mRNA in the cerebellum ($p < 0.01$) in comparison to control mice at 8 days after treatment. It is also noted that these levels returned to baseline values in the adulthood. Then, we evaluated behavioural performance in the T-Maze, a learning task that estimates procedural memory. At all ages, pST8SiaIII-treated mice showed a lower performance in the test session, being most evident at older ages ($p < 0.001$). Taken all together, we conclude that gene expression of ST8SiaIII is necessary for some cognitive tasks at early postnatal ages, since reduced levels impaired procedural memory in adult mice.

1. Introduction

Gangliosides that contain 2 or 3 residues with α2,8-linked sialic acid (Sia) chains are involved in several biological processes, including cell adhesion, signal transduction and cell differentiation, among others (Schauer et al., 1996; Angata and Varki, 2002). It has been previously demonstrated that α2,8-linked disialic acid (DiSia), as well as trisialic acid structures, occur in several glycoproteins in the mammalian brain (Sato et al., 2000; Inoko et al., 2010).

A coincidental pattern of expression has been observed for the DiSia epitope and ST8SiaIII mRNA (Rinflerch et al., 2012). Sialyltransferase ST8SiaIII is the enzyme responsible for catalysis of the transfer of a Sia residue to monosialylated glycoproteins, which occurs in the Golgi complex, where DiSia glycoconjugates are formed (Paul and Colley, 1989; Sato et al., 2000, 2002). We have previously demonstrated that DiSia is expressed at a constant level in the hippocampus, cortex and olfactory bulb, whereas its expression gradually decreases in the cerebellum throughout development (Rinflerch et al., 2012).

The cerebellum, together with other brain structures, is involved in the processing of working and procedural memories (Lalonde and Strazielle, 2003). Procedural memory is needed to use a previously learned skill (Okano et al., 2000).

Procedural and working memories can be examined in rodents using different behavioural tests, such as mazes (Lalonde and Strazielle, 2003; Deacon and Rawlins, 2006). Spontaneous alternation performance is the exploratory behaviour measurement most often studied in rodents because it reflects their natural instinct to explore the environment (Lalonde, 2002). One of the behavioural settings frequently used to examine this activity is the T-maze, which is sensitive for measuring performance associated with certain brain injuries and is especially well suited for working with mice (Whishaw and Tomie, 1996).

The aim of this work was to study the role of DiSia in the central nervous system (CNS) in mice. Given that DiSia levels decrease in the cerebellum in senile stages (Rinflerch et al., 2012), we inhibited

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the expression of ST8SiaIII mRNA in neonatal and adult mice using RNAi (RNA interference) and their performance was evaluated in a T-maze test. This test measures working and procedural memories, both of which are related to cerebellar function (Lalonde, 2002).

2. Materials and methods

2.1. Cell cultures and transfection

Cells of the murine neuroblastoma Neuro2A line were seeded at 1×10^6 cells for well (2 cm^2 area). Cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Argentina Ltd.). The plasmid kit was from Origene. Inc. (Cambridge, U.K.), with GFP (Green Fluorescent Protein) as a reporter gene and a RNAi sequence for mouse ST8SiaIII (Cat. n° TG502033), unspecific sequence and empty vector. Transfection of cells was made with Lipofectamine 2000™ (Invitrogen, Argentina Ltd).

Neuro2A cells were cultured in DMEM supplemented with 0.5 mg/ml of streptomycin sulfate, 100 units/ml of penicillin G, and 10% fetal bovine serum in a 5% CO_2–95% air humidified atmosphere at 37 °C.

A total of 2 μg/well plasmids were transfected with Lipofectamine2000™, following manufacturer's instructions. Culture medium was changed 24 h after transfection. Cells were induced for neural differentiation with 20 μM retinoic acid. The plasmids used were pST8SiaIII-RNAi (pST8SiaIII), pSI-RNAi (pSi) and pIS-RNAi (pIs). pST8SiaIII refers to an ST8SiaIII inhibitory sequence; pSi plasmid contains no insert; pIs contains an unspecific sequence (OriGene Technologies Inc., Cambridge, United Kingdom).

Cells were photographed daily for four days. At day 4, cells were fixed with paraformaldehyde 4% on PBS (PH: 7.2) for 8 min.

2.2. Animals

Five to twelve-hour-old C57BL/6 mice were injected intraperitoneal with 20 μL of mannitol (25%) using a Hamilton syringe (Ghodsi et al., 1999; Mastakov et al., 2001; Fu et al., 2003). Mice were anesthetized with dry ice situated on a stereotaxic frame (Cetin et al., 2006). Plasmids were administrated into the ventricular brain cavity (coordinates were in agreement with Pilpel et al., 2009). Each experimental group ($n = 10$) received 1.5 μg/μL of the corresponding plasmid (pST8SiaIII, pSi or pIs).

The total volume injected was 2 μL, according to previous assays (Pilpel et al., 2009).

Each group was randomly divided into subgroups. The first subgroup was analyzed by real-time PCR to assess mRNA levels of ST8SiaIII ($n = 30$ for each plasmid subdivided in three different ages), while a second subgroup was examined by Western-blot assay 8 days after injection ($n = 10$ for each plasmid). Finally, a third subgroup was submitted to the T-maze and then sacrificed for histological analysis.

Another group of mice (P30) ($n = 40$) was completely anesthetized with a combination of ketamine (Holliday-Skot, Buenos Aires, Argentina.) and Xilasine (*Rompun® Bayer*, Buenos Aires, Argentina) (75 mg/kg and 10 mg/kg, respectively) by intraperitoneal injection. Ventricle coordinates were obtained from Paxinos's atlas. (Bregma −0.7 mm dorsal, +1.20 mm lateral, +1.75 mm ventral) (Paxinos and Franklin, 2001).

Once the correct position was determined, the skull was perforated under sterile conditions, and 3 μg of plasmid was injected (pST8, pSi or pIs). As a control, another group of mice were injected with saline solution. Fifteen days after treatment, each group was submitted to the T-maze and then sacrificed for histological analysis.

This study was approved by our institution's animal care and use committee and followed NIH guidelines.

2.2.1. Real-time PCR

Reverse transcription was performed on 2 μg of total RNA, previously treated with RQ1 RNAse-free DNAse (Promega, Madison, USA) to eliminate possible contamination of genomic DNA. One microgram of treated RNA was used as template in a 20 μL volume cDNA synthesis reaction. Complementary DNA (cDNA) was synthesized using RT (ImpromII; Promega, Madison, WI) with oligo(dT) (Promega, Madison, WI) following the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR Green (Invitrogen Argentina Ltda), Platinum Taq Polymerase (Invitrogen Argentina Ltda.) and a LightCycler 2.0 Instrument (Roche Applied Science).

The protocol used was the following: 10 min at 94 °C and 40 cycles of 45 s at 94 °C, 30 s at the melting temperature (58 °C) and 10 s at 72 °C. In all cases, similar results were obtained when PCR was performed using cDNA. The GAPDH gene was used as a housekeeping gene. The primers for ST8SiaIII and β-actin genes were designed using the Primer3 program. Primers (Table 1) were designed according to the Gene Bank database.

The expression of mouse β-actin was used to standardize gene expression levels. Each sample was run in duplicate. Control experiments without template cDNA revealed no nonspecific amplification. To verify the identity of amplified DNAs, the sizes of the PCR products were checked on an agarose gel. The melting curves of all samples were always performed as a control of specificity.

An analysis of relative gene expression was performed using the Standard Curves method (Peinnequin et al., 2004; Bustin, 2000).

2.2.2. SDS Page

2.2.2.1. Protein isolation and electrophoresis. Brain samples (hippocampus, cerebellum, cortex and olfactory bulb) of 8-day-old mice from either the treated (pST8, pIs, pSi) or control group were homogenized in a mixture of 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM Triton X-100, and a proteinase inhibitors cocktail (1 μg/mL aprotinin, 2 μM leupeptin, 1 μM pepstatin) on ice. After centrifugation, proteins from the supernatant were quantified by the Bradford method, using a reactive Protein Assay (Bio-Rad, Argentina) on a Genesys 10S spectrophotometer (Thermo Scientific, U.S.A.).

Protein samples (80 μg) were dissolved in Laemmli buffer, incubated at 65 °C for 15 min and run on a 10% polyacrylamide electrophoresis gel, in a Miniprotean II System (Bio-Rad, Buenos Aires, Argentina) at 100 V for 1.5 h. Electrophoresis buffer contained Tris-base 0.125 M, glycine 0.96 M, SDS 0.05 w/v, pH 8.3. Page Ruler Plus Prestained Protein Ladder (Fermentas Inc., Maryland U.S.A.) was used.

Proteins were transferred to Polyvinylidene fluoride (PVDF) membranes (Millipore. Co, M.A. U.S.A.) for 2 h in a Miniprotean II System. Transfer buffer consisted of Tris-base 25 mM, glycine 192 mM, methanol 20% v/v, pH 8.6.

2.2.2.2. Western blot Immunostaining. PVDF membranes were blocked with 5% skim milk in TBS for 1 h at room temperature. Incubation with the primary antibody was performed overnight at 4 °C with agitation.

Antibody dilutions for anti-ST8SiaIII and anti- β-actin (both antibodies from Santa Cruz Biotechnology, Inc.) were 1:200 and 1:1000, respectively.

HRP-conjugated secondary antibody (dilution 1:1000) was incubated for 1 h at room temperature.

Table 1
Primer sequences.

Gene	Forward 5′ → 3′	Reverse 5′ → 3′
β-actin	GCCCAGAGCAAGAGAGGTA	AGAAGGTGTGGTGCCAGAT
ST8Sia III	GGCATTTCACCAGTCAGTCT	TATGTGGCAAAGCAGTCAGA

Immunostaining was revealed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and band intensity was quantified using ImageJ software (NIH, http://rsbweb.nih.gov/ij/). Results were expressed as relative optic density (Intensity/Area).

2.2.3. T-Maze

The behavioural testing was carried out according to previous publications (Deacon and Rawlins, 2006). Experimental groups consisted of: pST8SiaIII, pSi, pIs and Control mice, and were later evaluated on the T-maze using identical conditions.

At the time of testing, we used animals of the following ages: 45-day, 3-month, 6-month and 1-year-old. All animals of each group received a ventricular injection of plasmid combined with lipofectamine or saline solution on postnatal day 0. Another group of mice received a ventricular injection on postnatal day 30 and were evaluated 15 days later (Inject 30-day-old).

2.2.4. Immunohistochemistry

2.2.4.1. Immunofluorescence. Culture medium was removed, and Neuro2A cells were fixed with 4% paraformaldehyde for 8 min, and washed with PBS. Cells were then blocked with 10% Powerblock solution (Bio Genex, Inc., Oakland, CA.) and incubated with primary antibody: 10 μg/ml of S2–566 (Seikagaku Co., Japan) and 15 μg/ml of anti-ST8SiaIII at 4 °C for 20 h. After washing with PBS, cells were incubated for 1 h with anti-mouse IgM (7 μg/ml) and biotinylated immunoglobulins Multilink (Bio Genex, Inc., Oakland, CA.) for anti-Disia (S2–566), and anti-ST8SiaIII antibodies, respectively. Cells were incubated with Streptoavidin Texas Red (Vector Laboratories, Inc. Burlingame, CA.). Immunolabelling was visualized under a fluorescent microscope (Nikon eclipse E400).

The distribution of the ST8SiaIII epitope was also examined in mouse brain sections. Slides with 10 μm frozen brain sections were fixed with 4% paraformaldehyde at 25 °C for 5 min and washed with PBS. Brain sections were immunostained for ST8SiaIII and visualized under a fluorescent microscope, as described above. Nuclei were contrasted with Hoechst stain.

To quantify the density of labelling, we used ImageJ software. Ten points were randomly measured in each photo and the mean and the standard deviation for each sample group and positive control were determined. As a positive control, a Neuro2A cell culture was immunostained with the ST8SiaIII antibody 4 days after treatment with retinoic acid, in accordance to previous studies (Sato et al., 2002).

2.2.4.2. Klüver Barrera staining. Treated and control brains were embedded in paraffin. Slides with brain tissue were incubated in Luxol Fast blue solution (0.1% in 95% alcohol) overnight at 60 °C. The next day, tissue was rinsed in 95% alcohol to remove the excess

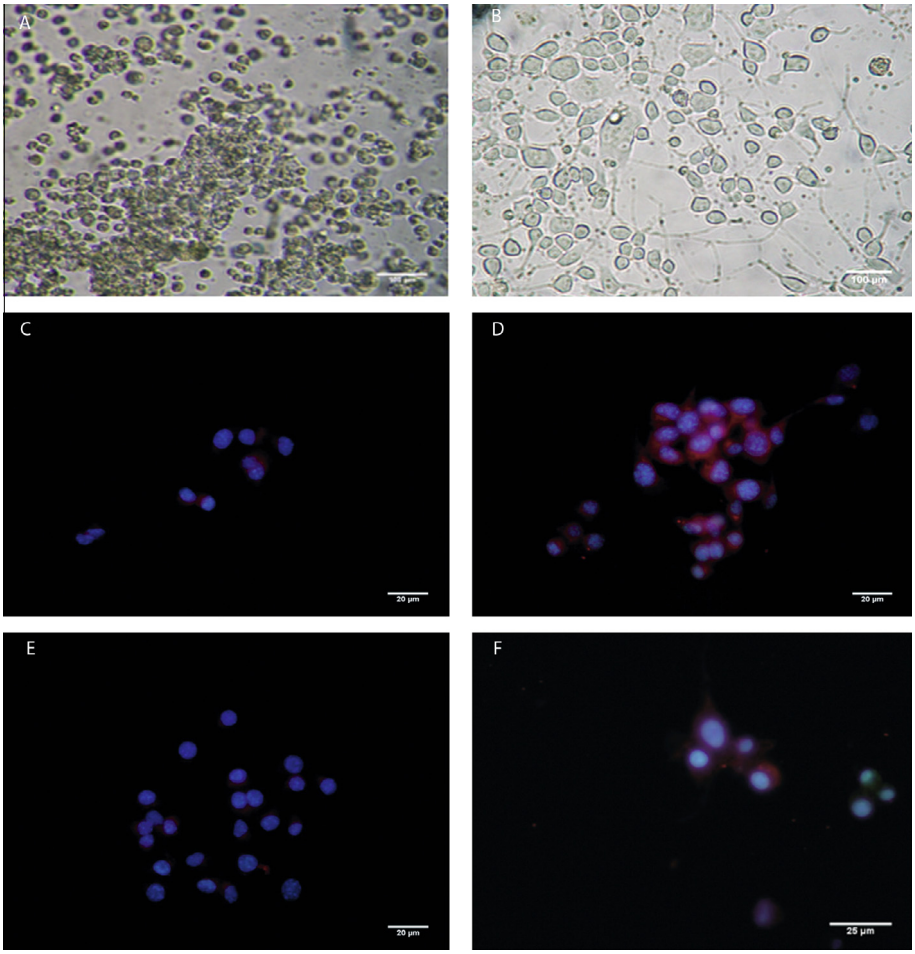

Fig. 1. Phase contrast imaging of Neuro2A cell culture. (A) Culture treated with pST8SiaIII and retinoic acid, (B) Culture treated with retinoic acid. Immunocytochemistry of ST8SiaIII and DiSia in Neuro2A cells (C) Culture treated with pST8SiaIII and retinoic acid, (D) Culture treated with retinoic acid (E) Culture without treatment (control culture). (F) Neuro2A cells transfected with pST8SiaIII (green, because of plasmid with GFP) and immunostained with S2–566 antibody (specific for DiSia). Secondary antibody was labelled with Texas Red fluorescent dye (red). Nuclei were stained with Hoechst (blue). Bar represents 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

colour. Differentiation was performed by immersion in a lithium carbonates solution (0.05% in distilled water), alternating with 70% alcohol until the desired colour was reached.

Cells were contrasted with neutral red stain (1% in distillate water).

2.3. Statistical analysis

To assess the quantitative differences among treated or control animals regarding different variables, an ANOVA test was performed using the software IBM SPSS Statistics 17 (http://www.spss.com) (Glantz, 2005).

3. Results

3.1. Neuro2A cells

As can be seen in Fig. 1A and C, culture cells treated with pST8SiaIII kept a round shape at day four, despite of the fact that they were induced to differentiate with retinoic acid. In contrast, cells that did not receive pST8SiaIII plasmid showed extension of neurites (Fig. 1B and D).

Furthermore, when Neuro2A cells treated with pST8SiaIII were immunostained with an antibody specific for DiSia (S2–566), they did not express the DiSia epitope or extension of neurites, indicating the correct incorporation of the plasmid and that cells did not differentiate. In all cases, green fluorescent protein (GFP) was used as a reporter gene (GFP+) (Fig. 1F).

3.2. Animals

3.2.1. Real-time PCR

As depicted in Fig. 2A, ST8SiaIII mRNA levels decreased significantly both in the cerebellum and hippocampus ($p < 0.01$) as well as in the cortex and olfactory bulb ($p < 0.05$) on day 8 after transfection. These results indicate that the plasmid was transfected and expressed in the brain and that the RNAi machinery was adequately activated to inhibit gene expression. ST8SiaIII mRNA levels returned to normal values, however, at 15 days and 3 months following plasmid transfection (Fig. 2B and C).

3.2.2. Western blot

ST8SiaIII protein expression was evaluated in different brain regions of 8 day-old mice of each experimental group. ST8SiaIII protein levels were significantly decreased in the cerebellum and olfactory bulb of pST8SiaIII-transfected animals (Fig. 3A and B); ($^{*}p < 0.05$). However no differences were observed in the hippocampus and cortex of pST8SiaIII-transfected mice compared to the control group (Fig. 3C and D).

3.2.3. T-maze

This behavioural task was used to evaluate procedural and working memories. The percentage of alternation between the left or right arm measures the exploratory activity of an animal. As shown in Table 2 and Fig. 4, pST8SiaIII-transfected animals showed a tendency to decrease alternation on the T-maze as they aged.

Mice that were transfected at birth and tested behaviourally 45 days later showed a lower percentage of alternation compared to control mice, but it was not statistically significant ($p > 0.05$ ANOVA test) (Table 2 and Fig 4). Their performance, however, was similar to senile control mice (13-months-old).

Three-month-old mice transfected at birth with pST8SiaIII performed poorly on the task compared to the control group (3-month-old) and senile control mice (13-month-old).

Three- and 6-month-old mice transfected with pST8SiaIII alternated arms less frequently compared to control animals. Three-month-old mice alternated arms at a frequency of 50% approximately, lower to what is expected (Deacon and Rawlins,

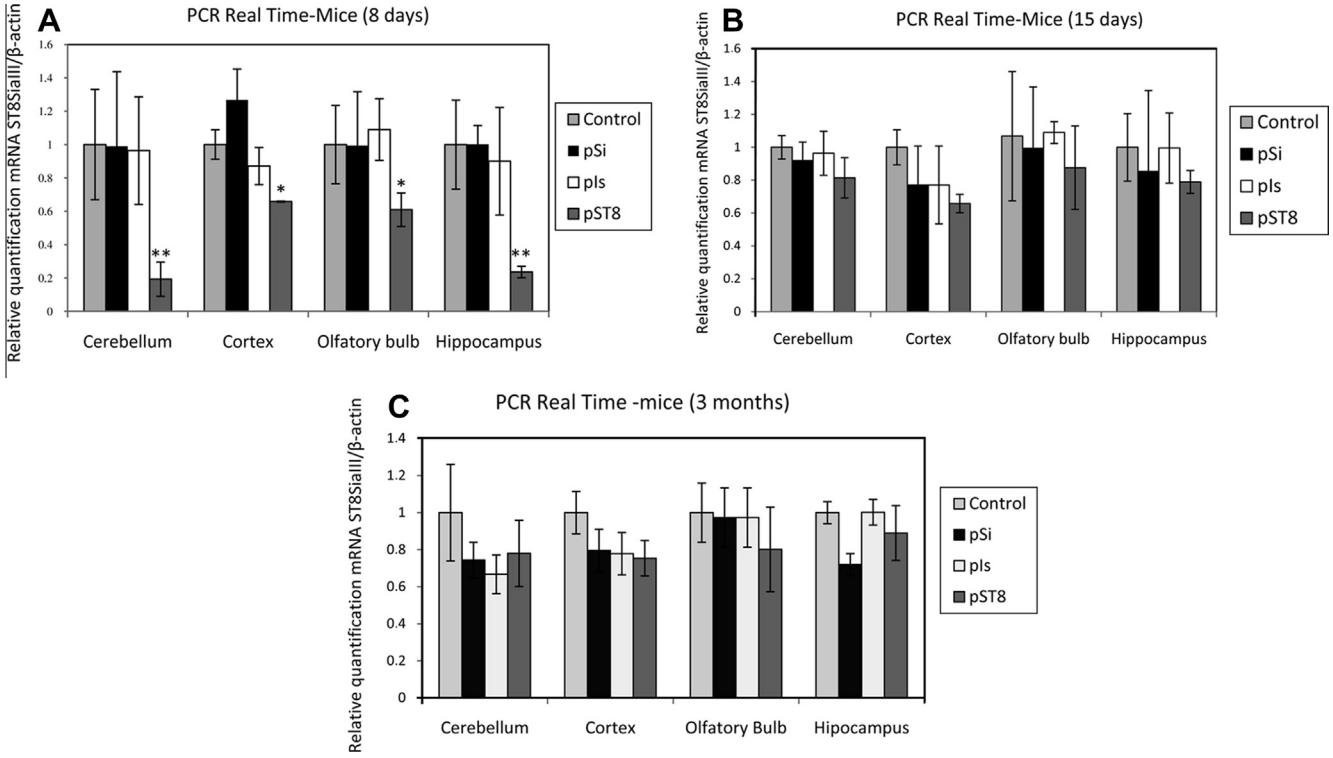

Fig. 2. Real-time PCR. Relative quantification of ST8SiaIII mRNA in cerebellum, cortex, olfactory bulb and hippocampus, of control, pSi-, pIs- and pST8SiaIII-transfected mice. (A) 8-day-old, (B) 15-day-old and (C) 3-month-old. Each group ($n = 10$), was transfected at birth. sample was run in duplicate. $^{*}p < 0.05$, and $^{**}p < 0.01$ ANOVA test.

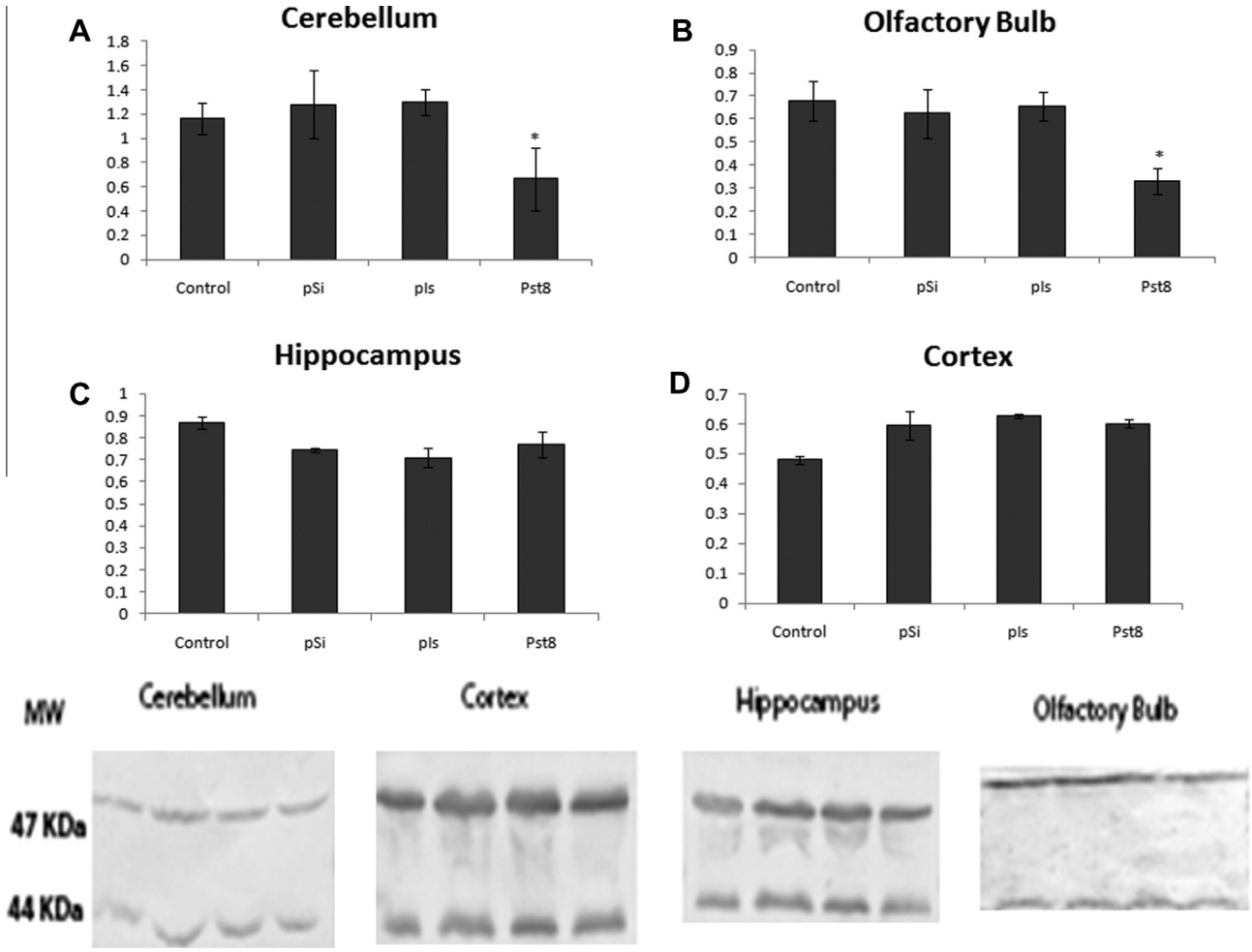

Fig. 3. Immunodetection and relative quantification of ST8SiaIII in the brains of control and, pSi-, pIs- and pST8SiaIII-transfected mice. Proteins subjected to electrophoresis were immunolabeled with anti-ST8SiaIII (44 kDa) and anti-β-actin (47 kDa) antibodies. Specific protein bands were visualized with DAB-based HRP reaction. (A) cerebellum, (B) olfactory bulb, (C) hippocampus, and (D) cortex. The quantification was relative to β-actin. $^{*}p < 0.05$ ANOVA test. Optical densities were measured using ImageJ software.

Table 2
Percentage of alternation on T-maze arms.

Mice	pST8SiaIII (%)	pSi (%)	pIs (%)	Control (%)
45-day-old	64	70	70	79
3-month-old	**53**	83	87	85
6-monts-old	**33***	69	83	76
Inject.30-day-old	64	70	73	80
13 month-old				65

2006), and had a poorer performance compared to senile control mice (13-months-old). Six-month old transfected mice had the worst performance ($p < 0.001$, ANOVA test) in the T-maze task among all groups (Table 2 and Fig 4).

Another group of mice, transfected with pST8SiaIII at postnatal day 30 and examined in the T-maze task 15 days after injection. These mice had a similar performance to the 45-day-old animals and senile control group ($p > 0.05$).

3.3. Immunostaining

3.3.1. Immunofluorescence

As shown in Fig 5, a decrease in labelling for the ST8SiaIII antibody was observed in the cerebellum of pST8SiaIII-transfected mice compared to control animals. Quantification of the ST8SiaIII staining density corroborated this observation (Table 3).

3.3.2. Klüver–Barrera staining

The Klüver–Barrera staining allows visualization of fibres and cells in the brain. We therefore stained brain slices from control (Fig. 6A and C) and pST8SiaIII-transfected mice (Fig. 6B and D).

We observed an irregular spongiform appearance in the white matter in most parts of the brain of pST8SiaIII-transfected animals, especially in the cerebellum (Fig. 6B and D).

4. Discussion

Based on previous studies, where it was shown that DiSia decreases in the cerebellum throughout the lifetime of mice (Rinflerch et al., 2012), in the present work we used RNA interference to study the role of DiSia in the mouse brain.

First, the correct functioning of ST8SiaIII RNAi was evaluated with the transfection of the plasmid in Neuro2A cells. Previously, Sato et al. demonstrated that Neuro2A cells express DiSia and that inhibition with S2–566 antibody blocked neurite extension (Sato et al., 2002). If ST8SiaIII is directly involved in the biosynthesis of DiSia, then inhibition of the ST8SiaIII mRNA by RNAi would interfere with the expression of DiSia, as it is shown in Fig. 1.

Similar to what happens with the expression of glycan structures on gangliosides, which quantities are dependent on the levels of enzyme glycosyltransferases (Ngamukote et al., 2007), here, we observed that the synthesis of glyco-epitope on glycoproteins is

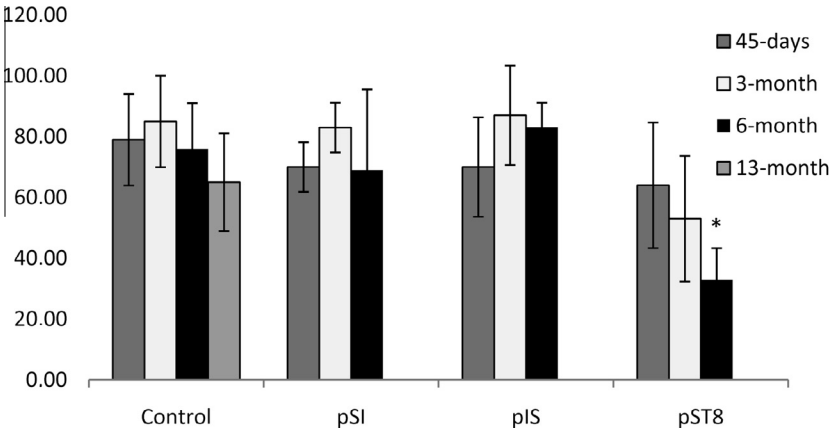

Fig. 4. Graphic representation of Table 3: Percentage of alternation on T-maze arms. 45 day-, 3-month-, 6-month- and 13-month-old mice. *p < 0.001 (ANOVA test). Each group (n = 10) was subject twice to the behavioural protocol.

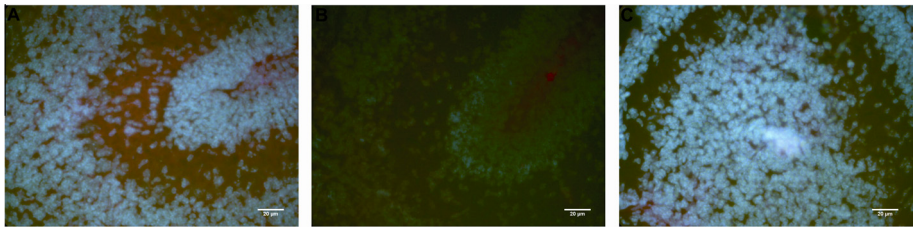

Fig. 5. Immunostaining of ST8SiaIII on cerebellum slices of 8 day-old control (A) and pST8SiaIII-transfected mice. (B) Negative control (incubation with PBS). (C) Cryosections were incubated with anti-ST8SiaIII as a primary antibody and labelled with Texas Red fluorescent dye (red). Nuclei were stained with Hoechst (blue). Bar represents 20 μm (10× magnification). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Results of the density analysis of positive marks for ST8SiaIII in the cerebellum.

	Positive (Neuro2A)	Control	pST8SiaIII treated
Mean +	82	145	55
Mean −	10	50	50
Total	72	95	5

Ten different sites on the same area in the cerebellum of Control and pST8SiaIII-transfected mice were randomly chosen. The final value was obtained from (mean +) − (mean −), where mean + refers to positive staining for ST8SiaIII and mean − refers to PBS in place of primary antibody. Positive control of the technique was a Neuro2A culture treated with RA for 4 days.

dependent on sialyltransferases in the cerebellum. This result provides more evidence that glycan biosynthesis is regulated at the transcriptional level (Nairn et al., 2008).

Previous reports have indicated that polysialic acids in the CNS are biosynthesized by ST8SiaII and ST8SiaIV sialyltransferases, and that their mRNA levels correlate with their enzymatic products: polysialic acids (Kojima et al., 1996; Nakayama et al., 1995). ST8SiaIII is a poor synthesizer of polysialic acids, but instead it is known to be involved in the formation of oligosialic acids (trisialic and disialic acid) (Angata et al., 2000; Inoko et al., 2010; Rinflerch et al., 2012). We previously reported a correlation between ST8SiaIII mRNA and DiSia epitope and a concomitant decreased expression in the cerebellum throughout the developing and aging mouse brain (Rinflerch et al., 2012).

Due to the relationship between cerebellar alterations and aging, and the significant decrease of ST8SiaIII mRNA and protein in our model (pST8SiaIII-transfected), we examined a cognitive task related to cerebellar function.

The present work demonstrates that mice treated with pST8SiaIII have reduced ST8SiaIII mRNA levels in the brain in the first 8 days following treatment, returning back to normal levels thereafter. It is important to note that at this stage of development, neural cells in the brain are still migrating to find their final position (Marín and Rubenstein, 2003; Angata et al., 2007; Chédotal and Rijli, 2009) and blocking the DiSia epitope with antibody prevents cells from extending their neurites (Sato et al., 2002). Thus, we hypothesized that the reduction of normal levels of DiSia in the brain by pST8SiaIII inhibition might alter the cell migration process.

The plasmid containing the ST8SiaIII-inhibiting sequence was injected into the IV ventricle of the mouse brain (Cetin et al., 2006; Pilpel et al., 2009). We chose this site of injection based on the fact that the origin of cells involved in the formation of the cerebellum is the ventricular neuroepithelium (Goldowitz and Hamre, 1998). This different site of cell origin for the cerebellum could be a possible explanation for the lower levels of ST8SiaIII observed in this work, in comparison to the olfactory bulb, cortex and hippocampus. These latter structures differ also in the track of migration and differentiation compared to the cerebellum.

Regarding the olfactory bulb, cortex and hippocampus we propose: (1) the turnover of ST8SiaIII mRNA could be higher in these brain regions compared to cerebellum, and the mRNA levels and sialyltransferase protein may be compensated by maintaining constant DiSia levels; (2) other sialyltrasferases are responsible for DiSia formation and ST8SiaIII has a different role in these brain regions; or (3) ventricular neuroepithelium cells incorporated all plasmids.

Further experiments will be needed to distinguish between these possibilities.

We focused our study on the cerebellar structure because there is a normal decrease in expression of both the DiSia epitope

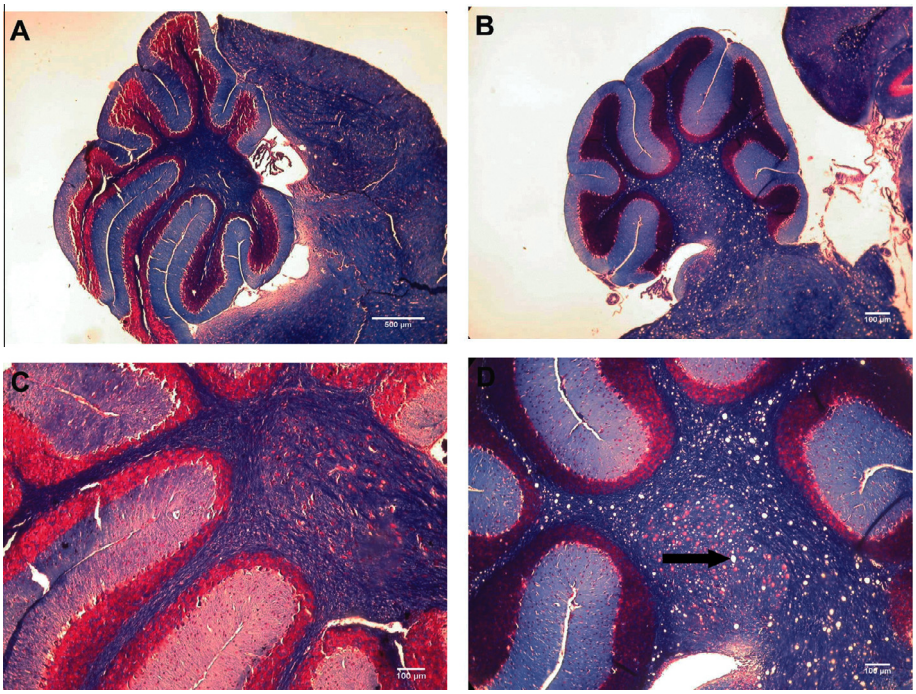

Fig. 6. Cerebellum sections subject to Klüver–Barrera stain, a method for the combined staining of cells (red), and fibers (blue) in the nervous system. (A) and (C): Control mice. (B) and (D): pST8SiaIII- transfected mice; Arrows indicate *holes* that microscopically confer a *spongiform aspect* to the tissue (A–B: 10× and C–D: 40× magnification). Bar represents A–B: 200 μm, and C–D: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and ST8SiaIII mRNA throughout development and aging in mice (Rinflerch et al., 2012). In addition, the cerebellum plays a role in behaviour and procedural memory (Manto and Marmolino, 2009a).

In the experiments presented here, we demonstrated that low levels of ST8SiaIII mRNA and DiSia early in development directly affect procedural memory, which could indicate the existence of underlying physiological disorders (Lalonde, 2002; Bastian, 2011).

Ataxia is characterized by failures in motor control (e.g. limb movement, gait and balance) and cognitive function, including spatial learning. Ataxia presents as a sporadic or as inherited form and can have different causes, generally based on combinations of biochemical and genetic disorders (Lalonde and Strazielle, 2003; Manto and Marmolino, 2009b).

Mice transfected with pST8SiaIII had a lower percentage of spontaneous alternation in the T-maze task (see Fig. 4), an effect that was more evident as animals aged.

Previous work has shown that patients with cerebellar ataxia have abnormal sialic acid metabolism, suggesting that sialylation of proteins is an important CNS process when impaired in the cerebellum (Mochel et al., 2009).

The relationship between clinical symptoms of ataxia and inherited diseases, such as those caused by lysosomal storage of gangliosides (GM1 and GM2 or Sadhoff) and hexosaminidase absence is well known (Manto and Marmolino, 2009a).

In addition, ataxia has been related to calcium channel mutations (Yue et al., 1997), the impairment of this subunit in the CNS and neuromotor nerves, and loss of channel activity throughout development (Chung et al., 2001). Calcium channels are surrounded with sialic acids in the extracellular matrix, glycolipids and glycoproteins of CNS cells.

Previous works on sodium and potassium channels on neural cells showed that polysialylation is important for their correct functioning (Zuber et al., 1992; Hall et al., 2011). Calcium channels do not undergo sialylation but the negative field provided by sialic acids is necessary for the correct functioning of voltage-gated calcium channels (Marengo et al., 1998). In fact, sialidase treatment causes a contraction of cardiomyocytes and deregulates the excitability of nervous cell membrane (Fermini and Nathan, 1991).

Based on this evidence, it is possible to hypothesize that the loss of sialic acids in the extracellular matrix could be underlying the cerebellar ataxia phenotype and/or others similar neurodegenerative diseases.

We therefore suggest, a potential relationship between the low DiSia levels in the cerebellum and the inactivation of voltage-gated calcium channels, which will be further studied in our laboratory. However, although not studied here, the loss of ST8SiaIII indirectly could have other physiological effects on the plasma membrane, such as variation on the myelination state, neurite outgrowth and cell migration (Sato et al., 2002; Janas and Janas 2011), with the consequent effect of the appearance of morphological abnormalities in the brain tissue; we found an irregular spongiform aspect in the white matter in most parts of the brain of pST8SiaIII-transfected animals, but especially in the cerebellum (see Fig. 6). A similar histological appearance has been reported previously in aged brains of patients with neurodegenerative diseases (Matalon and Michals-Matalon, 2000).

Although DiSia carriers in the mouse brain remain unknown, we can conclude that DiSia expression is crucial for proper CNS development and that the cerebellum has an important role in the processing of procedural and working memories.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuint.2013.07.013.

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