Differential expression of disialic acids in the cerebellum of senile mice

Adriana R Rinflerch, Valeria L Burgos, Alejandra M Hidalgo, Monica Loresi, and Pablo F Argibay

Instituto de Ciencias Básicas y Medicina Experimental - Hospital Italiano de Buenos Aires, Potosí 4240 8th floor, C1199ACL, Ciudad Autónoma de Buenos Aires, Argentina

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Introduction

Sialic acids (SAs) are commonly present as monosialyl residues at the non-reducing terminal end of glycoconjugates. Polysialic acid (PSA), one of the best known forms of SA, is attached to the neural cell adhesion molecule, NCAM (Angata et al. 2000). PSA promote changes in cell interactions and thereby facilitate changes related to neuroplasticity in the structure and function of the nervous system (Rutishauser and Landmesser 1996; Seki and Rutishauser 1998).

In some cases, α2,8-linked SA chains with two or three SA residues are linked to gangliosides and are involved in various biological processes, such as cell adhesion, cell differentiation, signal transduction and surface expression of stage-specific antigen (Schauer et al. 1996; Angata and Varki 2002).

It was 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). NCAM from adult mice are SA-containing glycoproteins. The physiological function and the specific anatomical distribution of those epitopes are not understood completely. However, based on in vitro studies, it is accepted that the diSia epitope is involved in neurite formation (Sato et al. 2002). Specifically, it was demonstrated that retinoic acid treatment increases the diSia epitope on the 100 kDa glycoprotein CD166, a cell adhesion molecule involved in neurite extension (Sato et al. 2002). In addition, it was demonstrated that the α2,8-sialyltransferase III (ST8Sia III) enzyme is responsible for the formation of the diSia epitope on CD166 (Sato et al. 2002). ST8Sia III synthesizes diSia from monosialylated glycoproteins and glycolipids (Yoshida et al. 1995) and might be important in the formation of the diSia glycoproteins on CD166 (Sato et al. 2002). Other sialyltransferases are involved in diSia synthesis: ST8Sia VI is shown to synthesize the diSia structure on glycopolymers and glycolipids. Other sialyltransferases are involved in diSia synthesis: ST8Sia I is responsible for diSia formation on glycolipids (Watanabe et al. 1996). However, the presence or even the role of these enzymes in the central nervous system has not yet been elucidated.

In the present work, we seek to elucidate the specific anatomical localization of the diSia epitopes in the central nervous system and the expression of the ST8Sia III gene involved in diSia transfer.

Based on the hypothesis that the diSia epitope is ubiquitously distributed in the central nervous system and that its
expression, along with the expression of the enzyme ST8Sia III is differentially expressed along life, the objective of the present work is to identify at a histological level the diSia epitope in different structures of the mouse brain (cortex, hippocampus, olfactory bulb and cerebellum) and investigate at a molecular level the differential expression of the ST8Sia III gene at various stages along life.

Results
Gene expression
Quantitative ST8Sia III gene expression on different brain regions was determined by real-time polymerase chain reaction (PCR). No significant differences for the ST8Sia III gene were found in the hippocampus, olfactory bulb or cortex in any of the groups. However, a significant decrease ($P = 0.01$, “$t$”-test) was found in the senile cerebellum (0.3480 ± 0.1803) compared with neonates (1 ± 0.1060; Figure 1A).

In order to analyze if the down-regulation of ST8Sia III was related to alterations at the synaptic level, the gene expression of synaptophysin, a synaptic vesicle glycoprotein, was analyzed in the cerebellum at different stages (Figure 1B). Both, in the neonate group and senile animals, the expression of the gene was diminished compared with the adult animals ($P < 0.01, F = 8.46$ analysis of variance between groups).

On the other hand, we observed that in the cerebellum of neonates the expression of the synaptophysin gene had an inverse correlation with that of the ST8Sia III gene ($r = -0.95, P < 0.05$). However, in adults and senile animals, the correlation was direct and elevated ($r = 0.84, P > 0.05; r = 0.83, P > 0.05$), indicating some relation between the expression of the two genes.

We also studied the gene expression of disialyltransferases ST8Sia I and ST8Sia VI in the same structures. ST8Sia I mRNA showed high levels of expression in the hippocampus of neonates and decreased levels in adults and senile. In contrast, in the olfactory bulb, the expression of the ST8Sia I gene was higher in the senile group compared with neonates and adults. We found very low levels of expression for the ST8Sia I gene in the cortex and cerebellum (Figure 1C). Regarding ST8Sia VI mRNA, we observed almost no expression for this gene in the neonate hippocampus but it was high in adults. In the olfactory bulb, cortex and cerebellum, a gradual increase in ST8Sia VI mRNA was found from low levels in neonates to high levels in senile animals (Figure 1D).

The ST8Sia II and ST8Sia IV polysialyltransferase genes were also analyzed because they synthesize both Neu5Acα2→8 Neu5Acα2→3Gal recognized by the S2-566 antibody. No staining differences in the hippocampus, olfactory bulb or cortex were observed but there was an important decrease in diSia labeling in the senile cerebellum compared with neonates (Table I; Figure 2A–D). N-Butyldeoxygalactonojirimycin (NB-DGJ)-treated mice showed the same pattern of S2-566 immunolabeling as the non-treated mice, which suggests that this labeling corresponds to glycoproteins (Figure 2D). This result is supported by the decreased labeling of GM1 observed in the whole brain of NB-DGJ-treated mice, when compared with non-treated animals (Figure 2F and G).

Discussion
The sialidation of glycoproteins and glycolipids is important for central nervous system development and function (Tsuji et al. 1983; Hildebrandt et al. 1998; Rutishauser and Landmesser 1996; Seki and Rutishauser 1998). Although the role and the distribution of PSA have been investigated, those of the oligosialic structures, such as disialic and trisialic acids, have not been fully examined. In this study, we describe the distribution of the diSia epitope, Neu5Acα2→8 Neu5Acα2→3Gal, and also the expression of the ST8Sia III gene. The results presented here suggest that the diSia epitopes recognized by S2-566 could be glycoproteins because after mice were treated with NB-DGJ, a ganglioside synthesis inhibitor, a similar pattern and the level of intensity of immunostaining were observed in the brain as the non-treated mice (Back et al. 2008), whereas the synthesis of GM1 gangliosides were markedly inhibited in the brain of treated mice (Figure 2F and G). Nevertheless, further experiments are needed to identify the diSia-carrier glycoproteins in the mouse brain.

Consistent with the expression of 2,8-linked diSia in the hippocampus, olfactory bulb, cortex and cerebellum from neonates to adult animals, we show that the mentioned areas consistently express the sialyltransferase ST8Sia III mRNA, which transfers one SA onto sialylated glycan acceptors.

Our results suggest a relation between the disialic epitope and the ST8Sia III enzyme, since when other disialyltransferases were studied (ST8Sia I and ST8Sia VI), both of these showed a distinct level in mRNA expression compared with diSia labeling.

Thus, at a transcriptional level, neural cells from different structures in neonates and adults generate 2,8-linked diSia at the cell surface. However, the cerebellum of senile animals shows a different pattern of both ST8Sia III transcriptional expression and of the corresponding glycoepitope (a significant decrease in the levels of expression of ST8Sia III and diSia epitopes). This result is very interesting; especially in relation to changes that ageing could produce in the cerebellum. Regarding the mechanism of sialidation, recent results show that patients with cerebellar ataxia present abnormalities in the metabolism of SAs, suggesting that the sialidation of key central nervous system proteins in the cerebellum is altered (Mochel et al. 2009). It is necessary to recognize that the mechanism(s) related to cerebellum neurodegenerative illnesses could have a direct relation to the changes that might cause aging. In this respect, we observed that ST8Sia III have equivalent levels of expression in the cerebellum of neonates and adults, with a gradual decrease until the senility stage. However, these results merit future lines of research to relate
neurodegenerative processes in the cerebellum caused by aging and the mechanisms of sialidation.

It is interesting to link the decreased gene expression of sialyltransferase with a relative decrease in the synaptic capacity in senile animals. In this sense, synaptophysin was used for quantification of synapses (Calhoun et al. 1996). We analyzed the gene expression of synaptophysin at different stages in the cerebellum and in the senile group, a similar pattern and also a correlation of gene expression between the expression of the ST8Sia III gene and the synaptophysin gene. This correlation of both enzymes in the cerebellum of senile animals could indicate some role of diSia at the synaptic level.

Fig. 1. Real-time PCR: relative quantification of mRNA. Black square, neonate group; gray square, adult group; dark grey square, senile group. (A) Relative quantification of ST8Sia III mRNA in the olfactory bulb, hippocampus, cortex and cerebellum of mice at different ages of postnatal development (*P < 0.05, “t”-test, between neonate and senile groups). (B) Relative quantification of synaptophysin mRNA in the cerebellum of mice at different ages of postnatal development of mice at different ages of postnatal development (*P < 0.05, “t”-test, between neonate and senile groups; **P < 0.01, “t”-test, between neonate and senile). (C) Relative quantification of ST8Sia I mRNA in the olfactory bulb, hippocampus, cortex and cerebellum of mice at different ages of postnatal development (*P < 0.05, “t”-test, between neonate and senile groups; **P < 0.01, “t”-test, between adults and senile). (D) Relative quantification of ST8Sia VI mRNA in the olfactory bulb, hippocampus, cortex and cerebellum of mice at different ages of postnatal development (*P < 0.05, “t”-test, compared with the neonate group). (E) Relative quantification of ST8Sia II mRNA in the olfactory bulb, hippocampus, cortex and cerebellum of mice at different ages of postnatal development (*P < 0.05, “t”-test, compared with the neonate group; **P < 0.01, “t”-test, compared with the neonate group). (F) Relative quantification of ST8Sia IV mRNA in the olfactory bulb, hippocampus, cortex and cerebellum of mice at different ages of postnatal development (*P < 0.05, “t”-test, compared with the neonate group; **P < 0.01, “t”-test, compared with the neonate group).
In relation to the other structures of the central nervous system, the expression of ST8Sia I, ST8Sia II, ST8Sia IV and ST8Sia VI on one hand and ST8Sia III on the other present notable differences in gene expression: while the first enzymes show a remarkably heterogeneous pattern of expression at different stages of life, ST8Sia III remains with a constant expression during development with levels statistically equivalent between adults and neonates. This would suggest that although other enzymes have a variable role in vital development with an important expression in postnatal level, ST8Sia III remains constantly expressed, indicating an important functional role in all life stages.

In conclusion, the differential pattern of expression of the ST8Sia III gene and also of the disialic epitope that shows a clear down-regulation in the cerebellum of senile animals, merit future lines of research regarding the roles of diSia in normal aging and in cerebellar diseases.

### Materials and methods

#### Animals and tissues

C57BL/6 mice of different stages were used: 5 days (neonate), 4 months (adult) and 10 months old (senile). Animals were raised in a temperature-controlled room (23°C) under a 12 h light:12 h dark photoperiod with lights on at 7 a.m. and light intensity of ~250 lux. Food (Purina rodent Chow, Ralston Purina, St Louis, MO) and water were provided ad libitum. This study was approved by our institution’s animal care and use committee and following NIH guidelines.

The brains were extracted after decapitation under anesthesia. Hippocampus, olfactory bulb, cortex and cerebellum were obtained through microdissection by trained people according to bibliography (Meredith and Arbuthnott 1993). In one group of animals (n = 6), RNA was extracted from the different tissues. In another equivalent group (n = 6), animals were anesthetized, and after the brains were extracted, they were embedded in Cryoplast (Biopack, Argentina), frozen in dry ice and cut in cryostat. The slices were stained to observe the anatomical localization of diSia by immunohistochemistry.

#### Real-time PCR

Reverse transcription was performed on 2 µg of total RNA, previously treated with RQ1 RNase-free DNase (Promega, Madison, WI) to eliminate the possible contamination of...
Table II. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5'→3'</th>
<th>Reverse 5'→3'</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>CTGGAGAAACCTGCCAAGTA</td>
<td>GTCCTCAGTGTAGCCCAAGA</td>
</tr>
<tr>
<td>ST8Sia I</td>
<td>CTGTTTCTGATGTTGGCCTTG</td>
<td>CATCTGTGTCCTGGAATCA</td>
</tr>
<tr>
<td>ST8Sia II</td>
<td>TTAGCCTGCGTAGACTTCC</td>
<td>GATAGAACGGCTCTGGAAGACA</td>
</tr>
<tr>
<td>ST8Sia III</td>
<td>GCCATTTCACAGTGACCTCT</td>
<td>TATGGGCAAAGCAGTCCGA</td>
</tr>
<tr>
<td>ST8Sia IV</td>
<td>CTTCACCATCGCTTTGTGT</td>
<td>GAAAGGGTGTGTTGTTAGA</td>
</tr>
<tr>
<td>ST8Sia VI</td>
<td>CGGCAAGCAGAAGAATTGAG</td>
<td>TCAAGTTAGCCTGCACTGGA</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>TGCGTGATGTCACCTTTTC</td>
<td>CATTICATCCAGCCACCT</td>
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genomic DNA. One microgram of treated RNA was used as a template in a 20 μL volume complementary DNA (cDNA) synthesis reaction. cDNA was synthesized using RT (ImpromII; Promega) with oligo(dT) (Promega) following the manufacturer’s instructions. Quantitative real-time PCR was performed using SYBR Green (Invitrogen Argentina Ltda.), Platinum Taq Polymerase (Invitrogen) and LightCycler 2.0 Instrument (Roche Applied Science, Mannheim, Germany).

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 as a sample. The Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) gene was used as a housekeeping gene. The primers for ST8Sia I, ST8Sia II, ST8Sia III, ST8Sia IV, ST8Sia VI, synaptophysin and GAPDH genes were designed using the Primer3 program. Primers (Table II) were designed according to the GenBank database.

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

Analysis of relative gene expression was performed using the standard curve method (Bustin 2000; Peinnequin et al. 2004).

Immunofluorescence microscopy

For immunofluorescence, the slides with the tissues were fixed with 4% paraformaldehyde at 25°C for 5 min, followed by washing with phosphate-buffered saline (PBS) at pH 7.2. The samples were then blocked with 10% Powerblock solution (Bio Genex, Inc., Oakland, CA.) and incubated with the primary antibodies S2-566 (Seikagaku Co., Japan) 10 g/mL or at 4°C for 20 h. After washing with PBS, the slides were incubated with a biotinilated IgM (Vector Laboratories, Inc., Burlingame, CA) secondary antibody at 25°C for 60 min, washed with PBS and incubated with Texas Red (Vector Laboratories, Inc. Burlingame, CA.), and then the nucleus was stained with Hoechst.

After washing with PBS, the samples were observed under a fluorescent microscope (Nikon eclipse E400). In order to evaluate if the staining corresponded to gangliosides or glyco-proteins, a group of animals (n = 4) was treated with NB-DGJ, a drug that specifically inhibits the glucosyleramide synthase. NB-DGJ was purchased from Toronto Research Chemicals, Inc. (North York, Ontario, Canada).

NB-DGJ solutions were prepared in sterile saline to yield a final concentration of 0.2 mg/μL. Post-natal C57BL/6 mice were injected daily intraperitoneally from p-0 to p-6 with either vehicle (0.9% saline) or NB-DGJ at 600 mg/kg body weight. Injections were performed using a Hamilton syringe (30 Gauge), and volumes ranged from 10 to 25 μL/mouse (Baek et al. 2008).

Tissues were obtained as described in Animals and Tissues section. The brains from NB-DGJ-treated vehicle or non-treated mice (neonatal, adult and senile) were separated from the skull and immersed in Cryoplast. The tissues were frozen on dry ice, stored at −80°C and sectioned on a cryostat (Microm HM 550) at 14 μm. To measure the density, we used the ImageJ software. Ten points were randomly measured in each photo and the mean and the standard deviation on each sample group and positive control were determined.

As a positive control, a Neuro2A cell culture was immunostained for the s2-566 antibody, 4 days after treatment with retinoic acid, in accordance to (Sato et al. 2002); (Figure 2).

NB-DGJ-treated mice were analyzed for GM1 composition with an anti-cholera toxin-B subunit conjugated with Alexa 555, according to previous work (Iglesias-Bartolomé et al. 2009).

Statistical analysis
To assess the quantitative differences among treated animals or controls regarding different variables, a software package was utilized (Glantz 2005).

All molecular biology and immunohistochemistry results were controlled with adequate positive and negative controls.

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Conflict of interest
None declared.

Abbreviations

cDNA, complementary DNA; diSia, disialic acid; NB-DGJ, N-butyldeoxygalactonojirimycin; NCAM, neural cell adhesion molecule; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PSA, polysialic acid; SA, sialic acid; ST8Sia molecule; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PSA, polysialic acid; SA, sialic acid; ST8Sia III, α2,8-sialyltransferase III.

References


