Glycobiology vol. 22 no. 3 pp. 411–416, 2012
doi:10.1093/glycob/cwr161
Advance Access publication on October 28, 2011

Differential expression of disialic acids in the cerebellum of senile mice

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Received on May 19, 2011; revised on October 5, 2011; accepted on October 25, 2011

It is known that disialic acids (diSia) are present in the mammalian brain. However, the precise anatomical distribution and the chronology of its expression along life are not well studied yet. It is accepted that the transfer of diSia in the brain is mediated mainly by the enzyme ST8Sia III (α2,8-sialyltransferase III). We studied the expression of diSia glycoepitopes and of the ST8Sia III gene in different structures of the mouse brain at different postnatal stages by immunohistochemistry and real-time polymerase chain reaction, respectively. C57BL/6 mice of different stages were used. Samples of hippocampus, olfactory bulb, cortex and cerebellum were processed for studies of molecular biology and immunohistochemistry. Histological analysis revealed an important decrease in diSia labeling in the senile cerebellum compared with other structures and stages ($P \ll 0.001$). In concordance with these results, a significant decrease in ST8Sia III gene expression was found in the cerebellum of senile animals ($P < 0.001$). These results suggest that diSia are constantly expressed but with differential expression in various areas of the mouse central nervous system. On the other hand, the concordance in the decreased expression of ST8Sia III and the diSia epitope in the cerebellum of senile animals suggest a role of diSia in this structure or, inversely, an influence of aging on the expression of diSia in the cerebellum. Further research in that direction could elucidate the roles of diSia in brain function in health and disease.

Keywords: brain / cerebellum / disialic acid / senile / ST8Sia III

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 glycotope on glycoproteins and glycolipids. Other sialyltransferases are involved in diSia synthesis: ST8Sia VI is shown to synthesize the diSia structure on *O*-linked glycan chains in glycoproteins (Takashima et al. 2002). Furthermore, it was demonstrated that 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

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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 → 8Neu5Ac and PSA epitopes. We found that ST8Sia II gene expression is constant in the cerebellum at all stages, whereas ST8Sia IV gene levels increase from neonates to adults and decrease again in the senile group (Figure 1E and F).

Distribution of the diSia epitope in the mouse brain

In concordance with molecular biology results, histological analysis revealed a similar pattern of expression for the diSia epitope, Neu5Acα2 → 8Neu5Acα2 → 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 → 8Neu5Acα2 → 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 (Baek 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

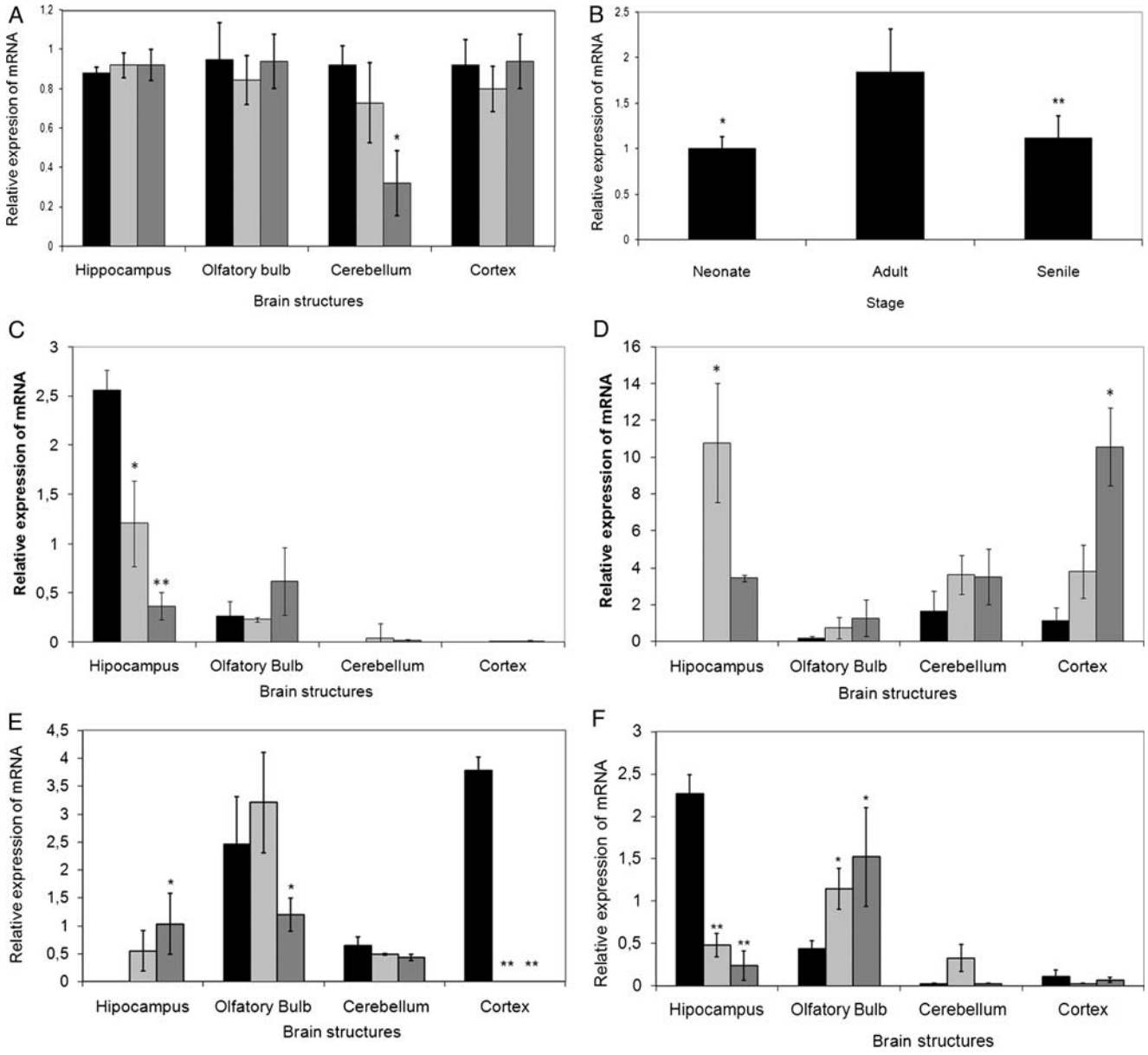

Fig. 1. Real-time PCR: relative quantification of mRNA. Black square, neonate group; gray square, adult group; dark grey square, senile group. (**A**) Relative quantification of ST8Sia III mRNA in the olfactory bulb, hippocampus, cortex and cerebellum of mice at different ages of postnatal development (*$P < 0.05$, "*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 (*$P < 0.05$, $t = -3.46$, "*t*"-test, between neonates and adults; **$P < 0.05$, $t = 2.72$, "*t*"-test, between adults 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, between neonate and senile groups; **$P < 0.01$, "*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).

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.

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.

Table I. Results of analysis of the density of positive marks for S2-566 in the mouse cerebellum at different stages of postnatal development.

	Positive (N2A)	Neonates	Adults	Senile
Mean+	124.680	220.780	102.175	84.825
Mean−	53.025	79.900	35.700	35.700
Total	121.655	140.88	66.4475	49.125

Ten different sites on the same area in the cerebellum were randomly chosen. The final value was obtained from (mean+) − (mean−), where mean+ is the positive staining for s2-566 and mean− the PBS in the place of the primary antibody. The positive control was a culture of N2A cells treated with RA during 4 days.

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

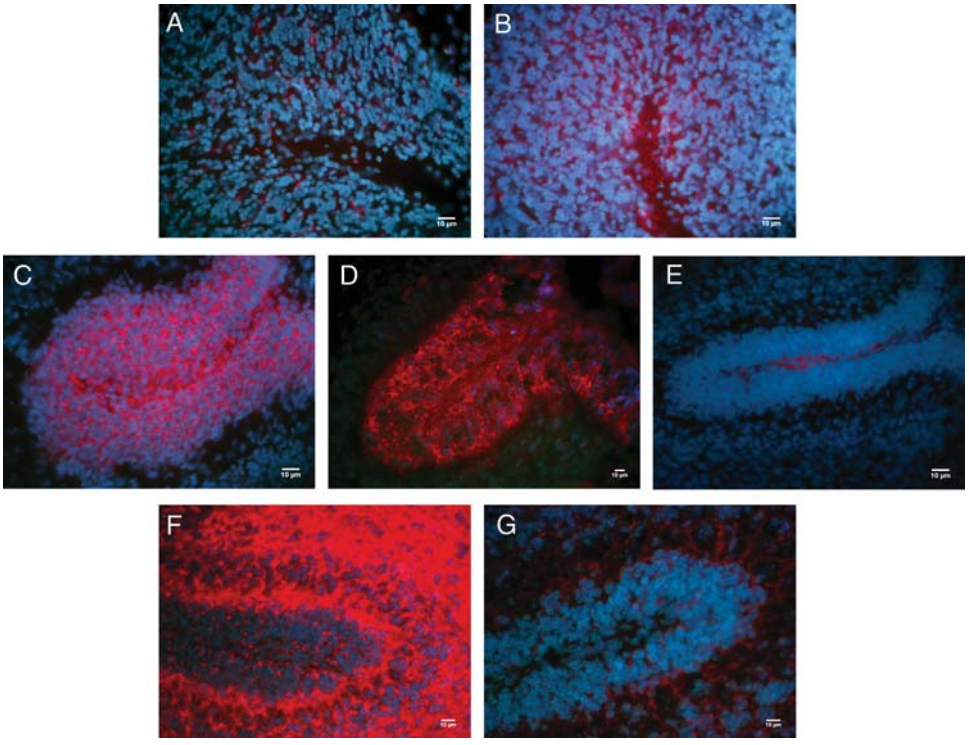

Fig. 2. Cerebellum slices of mice were immunostained with the S2-566 antibody. Nuclei were stained with Hoechst. (**A**) Senile, (**B**) adult, (**C**) neonate, (**D**) neonate treated with NB-DGJ and (**E**) neonate without the primary antibody (PBS). Cerebellum slices of neonates were immunostained with anti-cholera toxin Beta subunit Alexa 555. (**F**) Control mice and (**G**) NB-DGJ-treated mice. Bar represents 10 µm.

Table II. Primer sequences

Gene	Forward 5′ → 3′	Reverse 5′ → 3′
GAPDH	CTGGAGAAACCTGCCAAGTA	GTCCTCAGTGTAGCCCAAGA
ST8Sia I	CTGCTTTTGATGTTGGCTTG	CATCTGGTCCTCGAATCACA
ST8Sia II	TTAGCCCTGCGTACATTCTC	GATGAAAGGCTCTGGAGACA
ST8Sia III	GGCATTTCACCAGTCAGTCT	TATGTGGCAAAGCAGTCAGA
ST8Sia IV	CTTCACCCTCTGCTTTGTGT	GAAAGGGTGTGTGTGTGAG
ST8Sia VI	CGGCAAGCAGAAGAATATGA	TCATGTTAGTCCCCACTGGA
Synaptophysin	TGGGTCGATGTGACTTTTTC	CATTTCATCCAAGCCACCT

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 Glycerhaldheyde 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 glycoproteins, a group of animals ($n = 4$) was treated with NB-DGJ, a drug that specifically inhibits the glucosylceramide 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.

Funding

This work was supported by the Fundación para las Ciencias Básicas (FUCIBA), Buenos Aires, Argentina.

Acknowledgements

We would like to thank Dr. Ricardo Galimberti for technical assistance at the Department of Dermatology (Hospital Italiano de Buenos Aires), Dr. Jose Daniotti and Hugo Maccioni of Centro de Investigaciones en Química Biológica de Córdoba for kindly providing the anti-cholera antibody and Graciela Dabrowski and Emiliana Herrero of the Animal Facilities Area.

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 III, α2,8-sialyltransferase III.

References

Angata K, Suzuki M, McAuliffe J, Ding Y, Hindsgaul O, Fukuda M. 2000. Differential biosynthesis of polysialic acid on neural cell adhesion molecule (NCAM) and oligosaccharide acceptors by three distinct α2,8-sialyltransferases, ST8Sia IV (PST), ST8Sia II (STX), and ST8Sia. *J Biol Chem*. 275:18594–18601.

Angata T, Varki A. 2002. Chemical diversity in the sialic acids and related α-keto acids: An evolutionary perspective. *Chem Rev*. 102:439–469.

Baek R, Kasperzyk J, Platt F, Seyfried T. 2008. *N*-butyldeoxygalactonojirimycin reduces brain ganglioside and GM2 content in neonatal Sandhoff disease mice. *Neurochem Int*. 52:1125–1133.

Bustin S. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol*. 25:169–193.

Calhoun M, Jucker M, Martin L, Thinakaran G, Price D, Mouton P. 1996. Comparative evaluation of synaptophysin-based methods for quantification of synapses. *J Neurocytol*. 25:821–828.

Hildebrandt H, Becker C, Mürau M, Gerardy-Schahn R, Rahmann H. 1998. Heterogeneous expression of the polysialyltransferases ST8Sia II and ST8Sia IV during postnatal rat brain development. *J Neurochem*. 71:2339–2348.

Iglesias-Bartolomé R, Trenchi A, Comín R, Moyano AL, Nores GA, Daniotti JL. 2009. Differential endocytic trafficking of neuropathy-associated antibodies to GM1 ganglioside and cholera toxin in epithelial and neural cells. *Biochim Biophys Acta*. 1788:2526–2540.

Inoko E, Nishiura Y, Tanaka H, Takahashi T, Furukawa K, Kitajima K, Sato C. 2010. Developmental stage-dependent expression of an α2, 8-trisialic acid unit on glycoproteins in mouse brain. *Glycobiology*. 20:916–928.

Meredith G, Arbuthnott G. 1993. *Morphological Investigation of Single Neurons in Vitro. IBRO Handbook Series: Methods in Neurosciences*. Chichester (UK): John Wiley & Sons Ltd.

Mochel F, Sedel F, Vanderver A, Engelke U, Barritault J, Yang B, Kulkarni B, Adams D, Clot F, Ding J, et al.. 2009. Cerebellar ataxia with elevated cerebrospinal free sialic acid (CAFSA). *Brain*. 132:801–809.

Peinnequin A, Mouret C, Birot O, Alonso A, Mathieu J, Clarençon D, Agay D, Chancerelle Y, Multon E. 2004. Rat pro-inflammatory cytokine and cytokine related mRNA quantification by real-time polymerase chain reaction using SYBR green. *BMC Immunol*. 5:3.

Rutishauser U, Landmesser L. 1996. Polysialic acid in the vertebrate nervous system: A promoter of plasticity in cell-cell interactions. *Trends Neurosci*. 19:422–427.

Sato C, Fukuoka H, Ohta K, Matsuda T, Koshino R, Kobayashi K, Troy FA, 2nd, Kitajima K. 2000. Frequent occurrence of pre-existing α2→8-linked disialic and oligosialic acids with chain lengths up to 7 Sia residues in mammalian brain glycoproteins. Prevalence revealed by highly sensitive chemical methods and anti-di-, oligo-, and poly-Sia antibodies specific for defined chain lengths. *J Biol Chem*. 275:15422–15431.

Sato C, Matsuda T, Kitajima K. 2002. Neuronal differentiation-dependent expression of the disialic acid epitope on CD166 and its involvement in neurite formation in Neuro2A cells. *J Biol Chem*. 277:45299–45305.

Schauer R, Kelm S, Reuter G, Roggentin P, Shaw L. 1996. In Rosenberg A, editor. *Biology of the Sialic Acids*. New York: Plenum Press.

Seki T, Rutishauser U. 1998. Removal of polysialic acid-neural cell adhesion molecule induces aberrant mossy fiber innervation and ectopic synaptogenesis in the hippocampus. *J Neurosci*. 18:3757–3766.

Glantz S. 2005. *Primer of Biostatistics*. 6th ed. Mc Graw-Hill Medical Publishing Division. New York, N.Y.

Takashima S, Ishida H, Inazu T, Ando T, Ishida H, Kiso M, Tsuji S, Tsujimoto M. 2002. Molecular cloning and expression of a sixth type of α2,8-sialyltransferase (ST8Sia VI) that sialylates *O*-glycans. *J Biol Chem*. 277:24030–24038.

Tsuji S, Arita M, Nagai Y. 1983. GQ1b, a bioactive ganglioside that exhibits novel nerve growth factor (NGF)-like activities in the two neuroblastoma cell lines. *J Biochem*. 94:303–306.

Watanabe Y, Nara K, Takahashi H, Nagai Y, Sanai Y. 1996. The molecular cloning and expression of α2,8-sialyltransferase (GD3 synthase) in a rat brain. *J Biochem*. 120:1020–1027.

Yoshida Y, Kojima N, Kurosawa N, Hamamoto T, Tsuji S. 1995. Molecular cloning of Siaα2,3Galβ1,4GlcNAc α2,8-sialyltransferase from mouse brain. *J Biol Chem*. 270:14628–14633.