NeuroImage 58 (2011) 34–40

Contents lists available at ScienceDirect

NeuroImage

journal homepage: www.elsevier.com/locate/ynimg

Imaging of sialidase activity in rat brain sections by a highly sensitive fluorescent histochemical method

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ARTICLE INFO

Article history:
Received 4 March 2011
Revised 19 May 2011
Accepted 8 June 2011
Available online 16 June 2011

Keywords:
Fluorescent histochemical staining
Sialidase activity
Myelin
Hippocampus
Mossy fibers
Rat brain

ABSTRACT

Sialidase (EC 3.2.1.18) removes sialic acid from sialoglycoconjugates. Since sialidase extracellularly applied to the rat hippocampus influences many neural functions, including synaptic plasticity and innervations of glutamatergic neurons, endogenous sialidase activities on the extracellular membrane surface could also affect neural functions. However, the distribution of sialidase activity in the brain remains unknown. To visualize extracellular sialidase activity on the membrane surface in the rat brain, acute brain slices were incubated with 5-bromo-4-chloroindol-3-yl-α-D-*N*-acetylneuraminic acid (X-Neu5Ac) and Fast Red Violet LB (FRV LB) at pH 7.3. After 1 h, myelin-abundant regions showed intense fluorescence in the rat brain. Although the hippocampus showed weak fluorescence in the brain, mossy fiber terminals in the hippocampus showed relatively intense fluorescence. These fluorescence intensities were attenuated with a sialidase-specific inhibitor, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA, 1 mM). Additionally, the fluorescence intensities caused by X-Neu5Ac and FRV LB were correlated with the sialidase activity measured with 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (4MU-Neu5Ac), a classical substrate for quantitative measurement of sialidase activity, in each brain region. Therefore, staining with X-Neu5Ac and FRV LB is specific for sialidase and useful for quantitative analysis of sialidase activities. The results suggest that white matter of the rat brain has intense sialidase activity.

Introduction

Sialic acid is an acidic monosaccharide and plays crucial roles in various membrane functions in mammalian central nervous systems (Rutishauser, 2008; Schnaar, 2010). Sialidase removes sialic acid residues from sialoglycoconjugates, such as glycoproteins and glycolipids. Several different types of sialidase have been identified in mammalian tissues by their localization and enzymatic properties. Four types of mammalian sialidase (Neu1, Neu2, Neu3 and Neu4) have been cloned. Neu1, Neu2 and Neu3 are located at lysosomes (Bonten et al., 2009), cytosol (Hasegawa et al., 2000) and plasma membranes (Yamaguchi et al., 2006), respectively. Neu4 exists in the lysosomal lumen, mitochondria and intracellular membranes (Seyrantepe et al., 2004; Shiozaki et al., 2009). These four types of sialidase were reported to be expressed in mammalian brains (Shiozaki et al., 2009).

Extracellulary applied sialidase affects myelin–axon interactions, spinal axon outgrowth (Mountney et al., 2010) and cholinergic neurotransmission (Wieraszko and Seifert, 1984). Removal of polysialic acid (PSA), a polymerized structure of sialic acid with a degree of polymerization ranging from 8 to 400 (Kanato et al., 2008), by applying PSA-specific sialidase (EndoN) affects spatial learning (Becker et al., 1996), synaptic plasticity such as long-term potentiation and long-term depression (Kochlamazashvili et al., 2010; Muller et al., 1996), synaptogenesis (Dityatev et al., 2004), migration (Battista and Rutishauser, 2010) and innervations of GABAergic (Di Cristo et al., 2007) and glutamatergic (Seki and Rutishauser, 1998) neurons. Since extracellular sialidase activities are associated with many neural functions, it seems that enzyme activities on cell surface exhibited by endogenous sialidase could also affect neural

Abbreviations: 4MU-Neu5Ac, 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid; ac, anterior commissure; ACSF, artificial cerebrospinal fluid; AP, anterior–posterior from the bregma; alv, alveus; BSA, bovine serum albumin; cc, corpus callosum; cg, cingulum; cp, cerebral peduncle; cbw, cerebellar white matter; DANA, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid; dcw, deep cerebral white matter; dhc, dorsal hippocampus commissure; ds, dorsal subiculum; ec, external capsule; f, fornix; fi, fimbria; FITC, fluorescein isothiocyanate; fmi, forceps minor corpus callosum; FRV LB, Fast Red Violet LB; gcl, granular cell layer; h, hilus; hip, hippocampus; IC_{50}, 50% inhibitory concentration; ic, internal capsule; MBP, myelin basic protein; mpa, medial preoptic area; mrf, mesencephalic reticular formation; po, pons; PSA, polysialic acid; sc, superior colliculus; TBS-T, tris-buffered saline with 0.1% Tween-20; vp, ventral posterior thalamic nucleus; X-Neu5Ac, 5-bromo-4-chloroindol-3-yl-α-D-*N*-acetylneuraminic acid.

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doi:10.1016/j.neuroimage.2011.06.017

functions. However, the distribution of sialidase activity on cell surface in brain is poorly understood.

Previously, Saito et al. developed a novel fluorescent cytochemical method to detect sialidase activity of fixed neuroblastoma cells by using X-Neu5Ac as the substrate and an azo dye, FRV LB (Saito et al., 2002). After cleavage of X-Neu5Ac with sialidase, the compound X reacts with FRV LB, producing a water-insoluble fluorescent compound. This fluorescent method was also employed successfully for detection of sialidase activity of intact cultured cells. In the present study, we evaluated this highly sensitive fluorescent method for use in histochemical staining and examined the distribution of sialidase activity in the rat brain.

Materials and methods

Chemicals

The following products were purchased from the vendors indicated: X-Neu5Ac (Peptide Institute, Osaka, Japan); FRV LB and 4MU-Neu5Ac (Sigma-Aldrich, St. Louis, MO, USA); ZnAF-2 DA (Sekisui Medical, Tokyo, Japan); monoclonal antibody toward myelin basic protein (MBP, Cat. No. CP32) and sialidase from *Arthrobacter ureafacience* (Calbiochem, San Diego, CA, USA); DAPI (Invitrogen, Eugene, OR); fluorescein isothiocyanate-conjugated (FITC-conjugated) secondary antibody (Cat. No. 115-095-062, Jackson ImmunoResearch, West Grove, PA).

Spectrum analysis

The reaction mixture (200 μl) containing 1 mM X-Neu5Ac, 0.1 mg/ml FRV LB and 250 mU/ml sialidase from *Arthrobacter ureafacience* in 100 mM sodium acetate buffer (pH 4.8) was incubated at 27 °C for 15 min. The emission spectrum was collected at 550 nm excitation wavelength with a microplate reader Infinite M200 (Tecan, Männedorf, Switzerland).

Experimental animals

Male Wistar rats (8–9 weeks old) were purchased from Japan SLC (Shizuoka, Japan). They were housed under the standard laboratory conditions (23 ± 1 °C, 55 ± 5% humidity) and had access to tap water and diet *ad libitum*. The lights were automatically turned on at 8:00 and off at 20:00.

Statement on animal welfare

All experiments were performed in accordance with the Japanese Pharmacological Society guide for the care and use of laboratory animals, and the protocols were pre-approved by the Animal Ethical Committee of the University of Shizuoka.

Preparation of acute brain slices

For acute brain slices, Wistar rats were anesthetized with ether and decapitated. The brain of each rat was quickly removed and immersed in ice-cold artificial cerebrospinal fluid (ACSF, pH 7.3), containing 124 mM NaCl, 2.5 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$ and 10 mM D-glucose, to suppress excessive neuronal excitation and damage. Coronal brain slices (400 μm in thickness) were prepared by using a LinearSlicer PRO-7 (Dosaka, Kyoto, Japan) in ice-cold ACSF. ACSF used in the experiments was continuously bubbled with 95% O_2 and 5% CO_2.

In the case of staining for hippocampal mossy fiber terminals and nuclei, slices were incubated in ACSF containing 50 μM ZnAF-2 DA and 10 μg/ml DAPI at 27 °C for 30 min before use for sialidase activity imaging.

Imaging of sialidase activity

For imaging of extracellular sialidase activity on cell surface, acute brain slices were maintained in ACSF at room temperature for at least 60 min and then incubated with 400 μl ACSF (pH 7.3) containing 1 mM X-Neu5Ac and 0.1 mg/ml FRV LB at 27 °C for 1 h. Incubation chambers were continuously bubbled with 95% O_2 and 5% CO_2 during staining. Slices were washed three times with ACSF and transferred to IWAKI 3.5 mm glass based dishes (Asahi Glass, Tokyo, Japan) filled with ACSF. Fluorescent intensities were measured as brightness (arbitrary units) by using Photoshop CS4 (Adobe Systems, San Jose, CA).

Fluorescence was observed by using a fluorescence microscopy IX71 (Olympus, Tokyo, Japan) with filters (excitation/emission: BP530-550/BA575IF for sialidase activity imaging, BP460-495/BA510IF for ZnAF-2 DA, BP330-385/DM400 for DAPI). Background level of fluorescence for sialidase activity imaging was determined by incubating the brain slices in ACSF containing only 0.1 mg/ml FRV LB. In all observations with the fluorescence microscope, gain of a DP70 Digital Microscope Camera (Olympus) was set not to detect the background fluorescence.

Measurement of sialidase activity with 4MU-Neu5Ac

Acute coronal brain slices (400 μm in thickness) were cut into 2 mm square pieces with a razor blade in ice-cold ACSF. The pieces were incubated with 250 μl ACSF (pH 7.3) containing 10 μM 4MU-Neu5Ac at 27 °C for 90 min in a Nunc 96 microwell plates (Thermo Fisher Scientific). The supernatant (100 μl) was then transferred to a 96-well black microplate (Corning, NY, USA) filled with 50 μl of sodium carbonate (500 mM, pH 10.7) in each well. Intensity of 4MU-specific fluorescence (excitation, 355 nm; emission, 460 nm) was measured by a multilabel counter Wallac 1420 ARVOsx (PerkinElmer Life Sciences, Waltham, MA).

Immunohistochemistry

Serial brain sections (3 mm in thickness) next to the sections that were used for sialidase activity imaging were prepared. The brain sections were embedded into Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan). After being frozen, the brain sections were cut into 10-μm-thick sections at −20 °C with a Shandon Cryotome FE (Thermo Fisher Scientific, Waltham, MA). Sections were fixed with 4% paraformaldehyde for 15 min and then blocked in 5% bovine serum albumin (BSA) in tris-buffered saline with 0.1% Tween-20 (TBS-T, pH 7.4) for 15 min. After blocking with 5% BSA in TBS-T, the sections were incubated with a monoclonal antibody toward MBP (dilution 1:5) at 4 °C overnight and then rinsed in PBS. The slices were then reacted with FITC-conjugated secondary antibody (dilution 1:5) in 5% BSA in TBS-T at room temperature for 1 h. Fluorescence was observed by using a fluorescence microscope with filters (excitation/emission: BP460-495/BA510-550). Background level of fluorescence was determined by staining the brain slices with only the secondary antibody.

Results

We determined the optimal conditions for detection of sialidase activity with X-Neu5Ac and FRV LB. One mM X-Neu5Ac and 0.1 mg/ml FRV LB themselves showed low background fluorescence (Fig. 1). The sialidase from *Arthrobacter ureafacience* was added to a solution containing X-Neu5Ac and FRV LB, resulting in a remarkable increase in fluorescent intensity (Fig. 1). The fluorescence intensity acquired with a 550 nm excitation wavelength reached maximum at a 696 nm emission wavelength. We also determined the optimal FRV LB concentration in the range of 0.01 to 0.8 mg/ml by comparing the ratio of the signal fluorescence intensities to the background