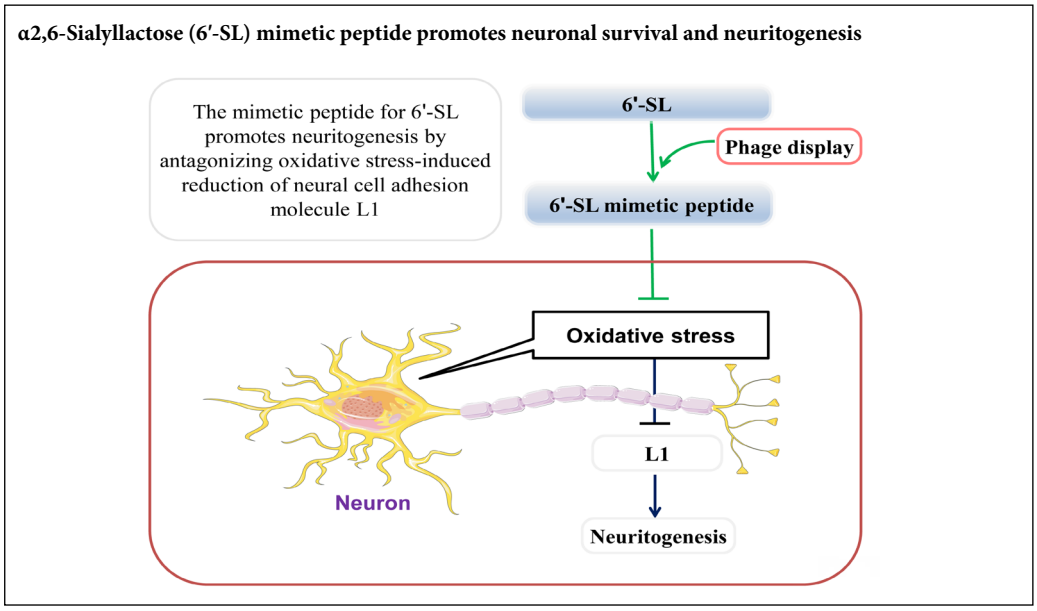
RESEARCH ARTICLE

A mimetic peptide of α2,6-sialyllactose promotes neuritogenesis

Shuang-Xi Chen[1, 2, #], **Jia-Hui He**[1, #], **Yong-Jian Mi**[1, 3, #], **Hui-Fan Shen**[1], **Melitta Schachner**[1, 4, *], **Wei-Jiang Zhao**[1, *]
1 Center for Neuroscience, Shantou University Medical College, Shantou, Guangdong Province, China
2 Department of Neurology, The First Affiliated Hospital of University of South China, Hengyang, Hunan Province, China
3 Department of Neurology, Chongqing Qijiang Renmin Hospital, Chongqing, China
4 Keck Center for Collaborative Neuroscience and Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, NJ, USA

Funding: *This work was supported by the National Natural Science Foundation of China, No. 81471279 and No. 81171138 (to WJZ), Talent Support Grant from Shantou University Medical College, China, No. 2501220118 (to WJZ), and the Li Kashing Foundation, No. LD030302 (to MS).*

Graphical Abstract



Correspondence to:
Melitta Schachner, PhD, schachner@stu.edu.cn;
Wei-Jiang Zhao, MD, PhD, weijiangnsc@163.com.

#These authors contributed equally to this work.

orcid:
0000-0002-3316-0778 (Melitta Schachner)
0000-0002-6556-2827 (Wei-Jiang Zhao)

doi: *10.4103/1673-5374.270313*

Received: *June 17, 2019*
Peer review started: *June 21, 2019*
Accepted: *July 28, 2019*
Published online: *December 10, 2019*

Abstract

Oxidative stress contributes to the pathogenesis of neurodegenerative diseases. With the aim to find reagents that reduce oxidative stress, a phage display library was screened for peptides mimicking α2,6-sialyllactose (6′-SL), which is known to beneficially influence neural functions. Using *Sambucus nigra* lectin, which specifically binds to 6′-SL, we screened a phage display library and found a peptide comprising identical sequences of 12 amino acids. Mimetic peptide, reverse peptide and scrambled peptide were tested for inhibition of 6′-SL binding to the lectin. Indeed, lectin binding to 6′-SL was inhibited by the most frequently identified mimetic peptide, but not by the reverse or scrambled peptides, showing that this peptide mimics 6′-SL. Functionally, mimetic peptide, but not the reverse or scrambled peptides, increased viability and expression of neural cell adhesion molecule L1 in SK-N-SH human neuroblastoma cells, and promoted survival and neurite outgrowth of cultured mouse cerebellar granule neurons challenged by H_2O_2-induced oxidative stress. The combined results indicate that the 6′-SL mimetic peptide promotes neuronal survival and neuritogenesis, thus raising hopes for the treatment of neurodegenerative diseases. This study was approved by the Medical Ethics Committee of Shantou University Medical College, China (approval No. SUMC 2014-004) on February 20, 2014.

Key Words: *central nervous system; cerebellar granule neurons; mimetic peptide; neural cell adhesion molecule L1; neuritogenesis; neurodegenerative disease; neuronal survival; oxidative stress; phage display; Sambucus nigra lectin; α2,6-sialyllactose*

Chinese Library Classification No. *R453; R741; Q816*

Introduction

The mammalian central nervous system is particularly vulnerable to oxidative stress, a phenomenon that is related to epigenetic factors, such as, for instance, low antioxidant levels in an organ with high oxygen consumption, surplus of iron ions, and high polyunsaturated fatty acid content (Ritchie et al., 2003; Farajdokht et al., 2017). Oxidative stress is hence one of the factors that cause neurodegeneration development and progression (Carri et al., 2015; Fischer and Maier, 2015). Oxidative stress-induced neuronal cell death underlies the progression of multiple neural diseases, such as Alzheimer's disease, schizophrenia and stroke (Hayashi-Takagi et al., 2014; Volpe and Paneni, 2015; Kamat et al., 2016; Chang et al., 2018). It is therefore reasonable to expect that anti-oxidative treatments may be applied as an appropriate strategy to ameliorate the symptoms of neurodegenerative diseases (Xu et al., 2017).

Sialic acid-containing glycans carried by glycoproteins and glycolipids have been broadly applied in clinical treatment of nervous system diseases (Ladeby et al., 2005; Varki, 2008; Du et al., 2009). One of the beneficial sialic acid-containing compounds is sialyllactose (SL), and it is composed of sialic acid and lactose, and classified into α2,3-SL and α2,6-SL dependent on the position at which the sialic acid is attached to lactose (Martin-Sosa et al., 2003). As a major source of sialic acid, SL functions as a component of gangliosides present on neuronal surfaces (Wang and Brand-Miller, 2003; ten Bruggencate et al., 2014), and it is crucial for brain development and function (Charbonneau et al., 2016; Liu et al., 2017). As an essential component of milk, it has been reported to display pro-active neural functions (ten Bruggencate et al., 2014; Tarr et al., 2015; Nagao et al., 2017; Jeon et al., 2018; Kang et al., 2018; Zehra et al., 2018). Difficulties in applying SL clinically relate to the problem that SL is not only cumbersome to purify from biological sources, but also very demanding to produce chemically. It is therefore important to obtain a reagent, such as a peptide, that specifically mimics the beneficial biological activities of SL and can be produced in sufficient amounts.

First introduced by Smith (1985), phage display technology has opened a powerful approach to obtain peptide ligands for almost any target. It can produce up to 1010 diverse peptides or protein fragments (Goldflam and Ullman, 2015; Galán et al., 2016; Messing, 2016). Peptide mimics for the human natural killer glycan (HNK-1) and α2,8-polysialic acid identified by phage display screening (Simon-Haldi et al., 2002; Torregrossa et al., 2004). They have shown to mimic the function of their glycan counterparts, with increased stability, added benefits of ease of production, and reduced generation cost (Ernst and Magnani, 2009; Magnani and Ernst, 2009).

The aim of this study was to use novel ways for identifying molecules that ameliorate oxidative stress. We investigated whether with the phage display method peptides can be identified that mimic the structure and function of 6′-SL and whether the selected mimetic peptide can protect cultured neurons against oxidative stress.

Materials and Methods

Animals

Twenty C57BL/6 specific-pathogen-free (SPF) female mice and ten C57BL/6 SPF male mice at the age of 4 weeks for the breeding of mice for primary culture of cerebellar granule neurons were obtained from the Guangdong Medical Laboratory Animal Center (licence No. SCXK (Yue) 2008-0002) and maintained on a 12-hour light/12-hour dark cycle at 25°C, with food and water ad libitum. All experimental protocols were approved by the Medical Ethics Committee of Shantou University Medical College, China (approval No. SUMC 2014-004) on February 20, 2014. Animals were maintained according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines for animal research (Kilkenny et al., 2010).

Screening for isolation of a mimetic peptide for 6′-SL

We used the Ph.D.TM−12 phage display peptide library (PDPL, New England BioLabs, Ipswich, MA, USA) where peptides are presented by the pIII protein on the pili of bacteriophage M13. Screening was performed as described (Simon-Haldi et al., 2002; Loers et al., 2019) with minor modifications. Briefly, heparin-binding 96-well plate wells (Corning, New York, USA) were coated overnight at 4°C with 15 μL of 1 mg/mL *Sambucus nigra* lectin (Cat# B-1305, Vector Laboratories, Burlingame, CA, USA) diluted in 135 μL $NaHCO_3$ (0.1 M, pH 8.6) to give a final concentration of 100 μg/mL. After blocking overnight at 4°C with 1% bovine serum albumin in phosphate-buffered saline (PBS, pH 7.3), the wells were then incubated with 1.5×10^{11} phages in Tris-buffered saline containing 0.1% Tween for 1 hour at room temperature. Wells were washed 10 times with Tris-buffered saline (pH 7.4) containing 0.1%, 0.3%, and 0.5% (v/v) Tween-20 (Cat# 9005-64-5, Sigma-Aldrich, St. Louis, MO, USA) in the first, second, and third rounds of panning, respectively. Phages were eluted using 100 μL of 1 M 6′-SL (Cat# 35890-39-2, Dextra Laboratories, Reading, England, UK) in each round of panning. After the first round of panning, eluted phages were amplified, and 2×10^{11} phages were taken for panning in the second and third rounds. Individual phage clones derived from the 3rd round of screening were isolated, and single-stranded phage DNA was purified for DNA sequencing (Sangon Biotech, Shanghai, China) to determine the peptide sequences. The following sequence was found in three phage clones: (H-PRHGKKPTNKRK-OH). The mimetic peptide, reverse peptide (H-KRKNTPKKGHRP-OH) and scrambled peptide (H-RPGHKKTPKNKR-OH) were synthesized by Hanhong Biochemical Company (Shanghai, China).

Phage binding enzyme-linked immunosorbent assay

To confirm that the obtained phages express a peptide mimicking 6′-SL, wells were pre-coated overnight at 4°C with 100 μL of 100 μg/mL Sambucus nigra lectin or $NaHCO_3$ as vehicle control. After blocking with 100 μL of 1% bovine serum albumin in PBS for 1 hour at room temperature, the wells were incubated with 1×10^{10} purified phages in PBS

for 2 hours at room temperature, with PBS as vehicle control. Wells were washed 6 times with Tris-buffered saline containing 0.1% Tween-20 before incubation with 100 μL of horseradish peroxidase-conjugated M13 antibodies (Cat# 27-9421-01, GE Healthcare, Piscataway, NJ, USA) in blocking solution for 1 hour at room temperature. The wells were then washed 6 times with Tris-buffered saline containing 0.03% Tween 20, and with 100 μL of 2,2′-azinobis-(3-et hylbenzthiazoline-6-sulphonate) (Cat# 30931-67-0, Sigma-Aldrich) finally. Absorbance was determined at 415 nm in a microtiter plate reader (Tecan Infinite® M1000 Pro, Tecan, Switzerland).

Competition enzyme-linked immunosorbent assay

To investigate whether the mimetic peptide can mimic 6′-SL, the mimetic or scrambled peptides were coated in 96-well plates at 20 μg/mL in PBS overnight at 4°C, and thereafter blocked with Carbo-free™ Blocking Solution (Cat# SP-5040, Vector Laboratories) for 2 hours at room temperature. After washing 5 times for 5 minutes each with PBS containing 0.1% Tween-20, wells were incubated overnight at 4°C with different dilutions (1:20, 1:100, 1:200, 1:500) of a 6′-SL/biotinylated Sambucus nigra lectin stock solution (Cat# B-1305-2, Vector Laboratories). After washing 5 times with PBS containing 0.1% Tween-20, wells were incubated with 100 μL horseradish peroxidase-coupled streptavidin (1:3000, Cat# BA1088, Boster, Wuhan, China) for 1 hour at 37°C. After washing 6 times with 0.1% PBS containing 0.1% Tween-20 for 5 minutes each at room temperature, wells were incubated with 2,2′-azinobis-(3-et hylbenzthiazoline-6-sulphonate) chromogenic substrate solution Substrate Chromogenic Solution (Cat# 37615, Thermofisher Scientific, Waltham, MA, USA) containing 30% H_2O_2 for 30 minutes at room temperature. Absorbance was determined with an enzyme-linked immunosorbent assay (ELISA) reader at 490 nm (Tecan Infinite® M1000 Pro).

Culture of SK-N-SH cells

Human neuroblastoma SK-N-SH cells were purchased from Chinese Type Culture Collection and cultured as described (Liu et al., 2013). Cells were treated with H_2O_2 as described (Zhang et al., 2017) with minor modifications. Briefly, 1×10^4 human neuroblastoma SK-N-SH cells were seeded into 96-well tissue culture plates (for cell viability assay) or 24-well tissue culture plates (for immunofluorescence staining of L1). Cells in wells of tissue culture plates were pretreated with 250 μM H_2O_2 for 2 hours prior to a 48-hour treatment with peptides at 20 μg/mL, when cell viability assay and L1 immunostaining were performed.

Culture of mouse cerebellar granule neurons

Cerebellar granule neurons were primary cultured from mice as described (Loers et al., 2019). Briefly, dissociated cells (2×10^4 cells in 100 μL Neurobasal A medium without B-27) were seeded into poly-D-lysine-coated 96-well tissue culture plates (for cell viability assay) or 24-well tissue culture plates (for neurite outgrowth assay). Cells were then pre-treated with 250 μM H_2O_2 for 2 hours prior to the 24-hour treatment with peptides at 20 μg/mL.

Cell viability assay

We evaluated cell viability using a Cell Counting Kit (CCK-8; Cat# HY-K0301, MedChem Express, China). Briefly, 10 μL CCK-8 solution was added to each well, respectively, and incubated at 37°C for 2 hours. Absorbance was then measured at 490 nm in a multi-well plate reader (Tecan Infinite® M1000 Pro).

Neurite outgrowth assay

Cells were fixed by adding 100 μL of 4% formaldehyde in PBS and then stained with Crystal Violet Staining Solution (Cat# C8470, Solarbio, Beijing, China). Neurite lengths of individual cerebellar granule neurons were measured at a 40× magnification by an Image Analysis System (IX-51, Olympus, Tokyo, Japan). Only neurites with a length of at least one diameter of the cell body and without contact with other cells were measured. Approximately 50 cells were counted per experiment and per experimental group, and each experiment was repeated for three times.

Immunofluorescence staining

After rinsing with PBS, human neuroblastoma SK-N-SH cells were fixed with 4% formaldehyde in PBS for 10 minutes, and then washed with PBS. 10% normal donkey serum was used to block the unspecific binding sites (Cat# 017-000-001, Jackson ImmunoResearch Laboratories) in PBS at room temperature for 40 minutes. Cells were then incubated with mouse monoclonal antibody against L1 (1:200; Cat# No. MAB777, R&D Systems, Minneapolis, MN, USA) overnight at 4°C. Samples were rinsed three times with PBS for 5 minutes each at room temperature and incubated with donkey anti-mouse secondary antibody conjugated to Alexa Fluor® 488 (1:500, Cat# A28175, Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature. The coverslips were mounted using the ProLong® Gold Antifade reagent with 4′,6-diamidino-2-phenylindole (Cat# P36935, Gibco; ThermoFisher Scientific). Confocal images were acquired using the FV10-ASW software (Ver. 01.01) under an Olympus confocal system (FV-1000, Olympus, Tokyo, Japan). The immunofluorescence intensity indicating the level of L1 was measured using ImageJ 5.0.

Statistical analysis

GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analyses. Data are expressed as means ± SEM. One-way analysis of variance was performed followed by the *post-hoc* Bonferroni test, and $P < 0.05$ was considered statistically significant.

Results

Identification of a mimetic peptide

To identify a mimetic peptide, a phage display library containing 1×10^9 different filamentous phages presenting 12-mer peptides on phage coat protein pIII was screened. The

eluted phage clones were probed for their binding to the 6′-SL-specific Sambucus nigra lectin by ELISA, with the phage clones M1, M5, M12 and M14 clones showing best binding (**Figure 1**). Clones were sequenced and found to contain identical insert sequences, except for M5 which failed in sequencing (**Table 1**).

To verify mimic specificity for 6′-SL, the peptides with the best binding in the phage ELISA were synthesized. Their reverse and scrambled counterparts were also synthesized. With increasing ratios of 6′-SL over biotinylated Sambucus nigra lectin in the pre-incubation solution, the binding of the mimetic peptide to the lectin was competitively decreased, indicating that the mimetic peptide was structurally similar to 6′-SL. The scrambled peptide did not show any binding in comparison to the blank control groups (**Figure 2**).

Mimetic peptide increases the viability of H_2O_2-stressed SK-N-SH cells and cerebellar granule neurons

To explore the effect of the mimetic peptide on neuronal survival *in vitro*, cell viability assays were performed with SK-N-SH cells or cerebellar granule neurons after pre-treatment with 250 μM H_2O_2 for 2 hours before adding the mimetic, reverse or scrambled peptides each at a concentration of 20 μg/mL for 48 hours or 24 hours. In comparison with the vehicle control group, H_2O_2 treatment reduced survival of SK-N-SH cells. The mimetic peptide increased survival of H_2O_2 stressed SK-N-SH cells, whereas the reverse and scrambled peptides did not decrease cell death (**Figure 3A**). A similar result was observed for viability of cerebellar granule neurons (**Figure 3B**).

Mimetic peptide increases neurite length of H_2O_2-stressed cerebellar granule cells

To verify the effects of the mimetic peptide on neurite outgrowth under oxidative stress, neurite outgrowth of cerebellar granule cells was determined. In comparison with the vehicle control group, H_2O_2 treatment reduced the lengths of neurites. The mimetic peptide increased neurite lengths compared with the H_2O_2-treated group, whereas the reverse and scrambled peptides did not increase neurite lengths (**Figure 4**).

Mimetic peptide increases L1 expression in H_2O_2-stressed SK-N-SH cells

To study the effects of the mimetic peptide on the expression of the neural cell adhesion molecule L1 under oxidative stress, we performed immunofluorescence staining on SK-N-SH cells. In comparison with the control group, H_2O_2 treatment reduced L1 expression. Compared with the H_2O_2-treated group, the mimetic peptide increased L1 expression under H_2O_2 conditions, whereas L1 levels did not change after treatment with the reverse or scrambled peptides (**Figure 5**).

Discussion

Recent studies have verified the potential of mimetic peptides for drug design and therapy (Mehanna et al., 2010; Masand et al., 2012; Prost et al., 2012). Screening of phage display libraries benefits many molecular and cellular functions, as evidenced by peptide drug discovery (Hamzeh-Mivehroud et al., 2013), identification of biomarkers (Ghoshal et al., 2016), isolation of high-affinity antibodies (Azzazy and Highsmith, 2002), and vaccine development (Aghebati-Maleki et al., 2016). Regarding the mimicry of pro-active carbohydrates, mimetic peptides for α2,8-polysialic acid promote functional recovery following spinal cord injury in mice (Marino et al., 2009; Mehanna et al., 2010) and accelerate myelination and functional recovery following peripheral nerve injury in the mouse (Mehanna et al., 2009). In the present study, we found a peptide that mimics not only the structure of 6′-SL, but enhances neuronal survival and neuritogenesis, accompanied by increased expression of the regeneration-beneficial cell adhesion molecule L1.

Previous studies have shown degenerative changes in the cerebellum in Huntington's disease, Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Colloby et al., 2014; Tan et al., 2014; Burciu et al., 2015; Wolf et al., 2015). Thus, cerebellar granule neurons have been widely

Table 1 Sequences of phage-encoded peptides that bound to Sambucus nigra lectin

Clone names	Codons (5′–3′)	Peptide sequences (N′–C′)
M10	GCT GAT GCT AGG CCG TGG TGG AAG AGT CAG GGT TTT	ADARPWWKSQGF
M9	TTT CCG TAT CCT ATG AAT AAG CAG ACT AAT GGT ACT	FPYPMNKQTNGT
M2	AAT CAT CGG AAG GTT AGT AGG CAT GCG ACT CAT TTT	NHRKVSRHATHF
M6	AAT CAT CGG AAG GTT AGT AGG CAT GCG ACT CAT TTT	NHRKVSRHATHF
M8	AAT CAT CGG AAG GTT AGT AGG CAT GCG ACT CAT TTT	NHRKVSRHATHF
M13	AAT CAT CGG AAG GTT AGT AGG CAT GCG ACT CAT TTT	NHRKVSRHATHF
M1	CCG AGG CAT GGG AAG AAG CCT ACG AAT AAG AGG AAG	PRHGKKPTNKRK
M12	CCG AGG CAT GGG AAG AAG CCT ACG AAT AAG AGG AAG	PRHGKKPTNKRK
M14	CCG AGG CAT GGG AAG AAG CCT ACG AAT AAG AGG AAG	PRHGKKPTNKRK
M7	ACG GTT GAT TCG GCT AGT CTT TTG CAG AGT CGT ACT	TVDSASLLQSRT
M3	TGG GGG TTT CAT TGG CCG GTG TAT CCT CCG TCT AGG	WGFHWPVYPPSR
M4	Empty carrier	
M11	Empty carrier	
M5	Failed to be sequenced	

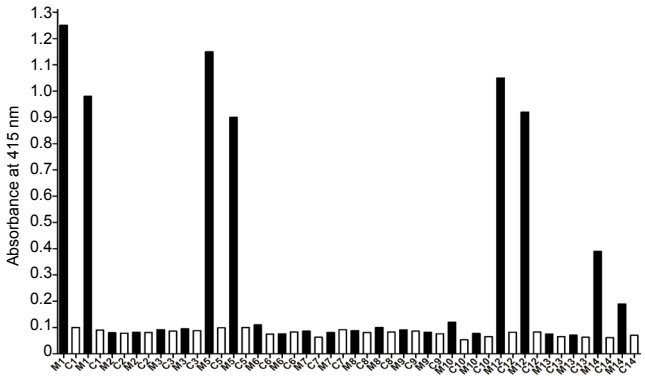


Figure 1 Identification of phages binding to *Sambucus nigra* lectin.
After three panning rounds, duplicate samples of phages M1, M5, M12, and M14 were identified to bind to lectin with high affinity by enzyme-linked immunosorbent assays (black bars). Vehicle controls, also in duplicate, are indicated by white bars.

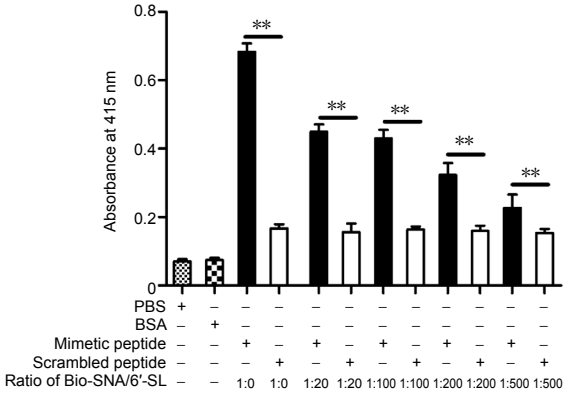


Figure 2 Enzyme-linked immunosorbent assay showing concentration-dependent inhibition of biotinylated *Sambucus nigra* (Bio-SNA) binding to α2,6-sialyllactose (6′-SL) mimetic peptide-coated substrate by 6′-SL.
Negative controls were substrate coated with scrambled peptide, bovine serum albumin (BSA) and phosphate-buffered saline (PBS). Data are expressed as the mean ± SEM. $^{**}P < 0.01$ (one-way analysis of variance followed by the Bonferroni *post-hoc* test). The experiment was repeated three times.

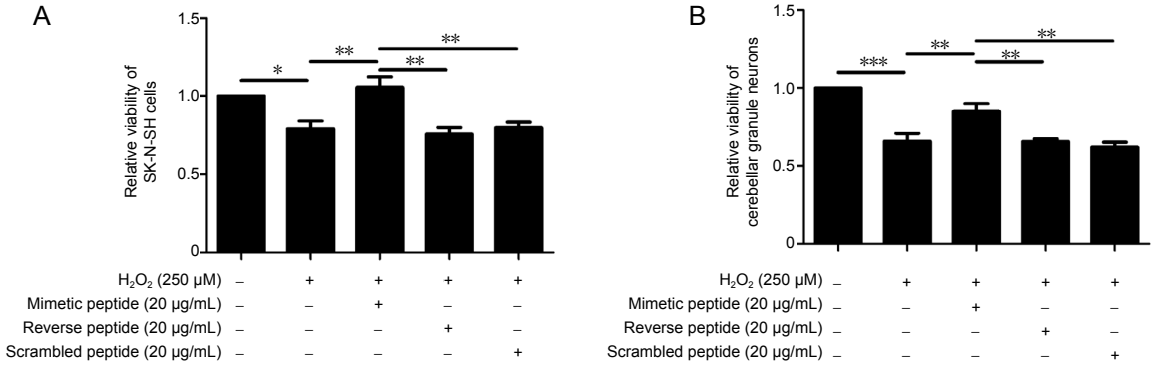


Figure 3 The α2,6-sialyllactose (6′-SL) mimetic peptide, but not its reverse or scrambled counterparts, enhances survival of SK-N-SH cells (A) and mouse cerebellar granule neurons (B) under H_2O_2-induced oxidative stress.
6′-SL mimetic peptide: H-PRHGKKPTNKRK-OH; 6′-SL reverse peptide: H-KRKNTPKKGHRP-OH; 6′-SL scrambled peptide: H-RPGHKKTP-KNKR-OH. Data are expressed as the mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (one-way analysis of variance followed by the Bonferroni *post-hoc* test). The experiment was repeated five times.

used to study apoptosis in neurodegenerative diseases (Chen et al., 2009), since they are particularly vulnerable to oxidative insults due to their membranes being mainly composed of polyunsaturated fatty acids, which are substrates for reactive oxygen species (Wang et al., 2017). Neuroblastoma cells have also served for studying neural functions because of their similarity to mature neural stem cells that can be differentiated into neurons and astrocytes (Ross et al., 2003; Ross and Spengler, 2007). Also, they have been paradigmatic for the study of neurons in cell culture. In a previous study, the cell viability of SK-N-SH cells was decreased in a dose- and time-dependent manner in response to the treatment of H_2O_2 (Zhang et al., 2017). In our present research, we treated the SK-N-SH cells and cerebellar granule neurons with 250 μM H_2O_2 for only 2 hours to induce mild cell death, which is typical to early-onset neurodegenerative diseases. Indeed, both human neuroblastoma and mouse cerebellar granule neurons responded favorably to the 6'-SL mimetic peptide in terms of cell survival.

Neurite outgrowth is an important feature of neuronal plasticity and regeneration after injury (Khodagholi et al., 2012). Previous studies have reported that a tenascin-C mimetic peptide-coupled nanofiber gel can promote neurite outgrowth and cell migration of neurosphere-derived cells (Berns et al., 2016). A mimetic peptide of the S-100 protein attenuates the progression of neuropathy and enhances peripheral nerve regeneration in myelin protein P0 null mice (Moldovan et al., 2013). A peptide mimetic targeting the neural cell adhesion molecule NCAM can promote neural plasticity and spatial learning in the hippocampus (Kraev et al., 2011). Neuroplastin-65 and a mimetic peptide derivative of its homophilic binding site can modulate neuritogenesis and neuroplasticity (Owczarek et al., 2011). Phage display libraries have also been useful in selecting peptides that bind to targets of choice: chondroitin-4-sulfate-binding peptides enhance neurite outgrowth on inhibitory chondroitin sulfate proteoglycans (Loers et al., 2019).

We used the neural cell adhesion molecule L1 as an indi-

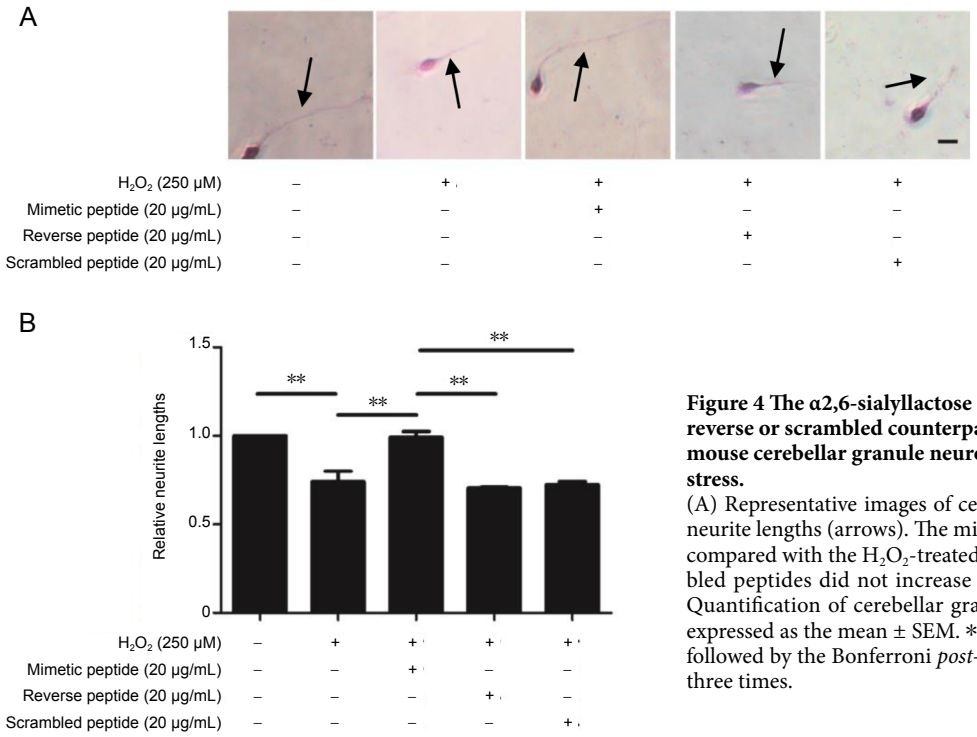


Figure 4 The α2,6-sialyllactose (6′-SL) mimetic peptide, but not its reverse or scrambled counterparts, enhances neurite outgrowth of mouse cerebellar granule neurons under H_2O_2-induced oxidative stress.

(A) Representative images of cerebellar granule neurons with different neurite lengths (arrows). The mimetic peptide increased neurite lengths compared with the H_2O_2-treated group, whereas the reverse and scrambled peptides did not increase neurite lengths. Scale bar: 20 μm. (B) Quantification of cerebellar granule neuron neurite lengths. Data are expressed as the mean ± SEM. $^{**}P < 0.01$ (one-way analysis of variance followed by the Bonferroni *post-hoc* test). The experiment was repeated three times.

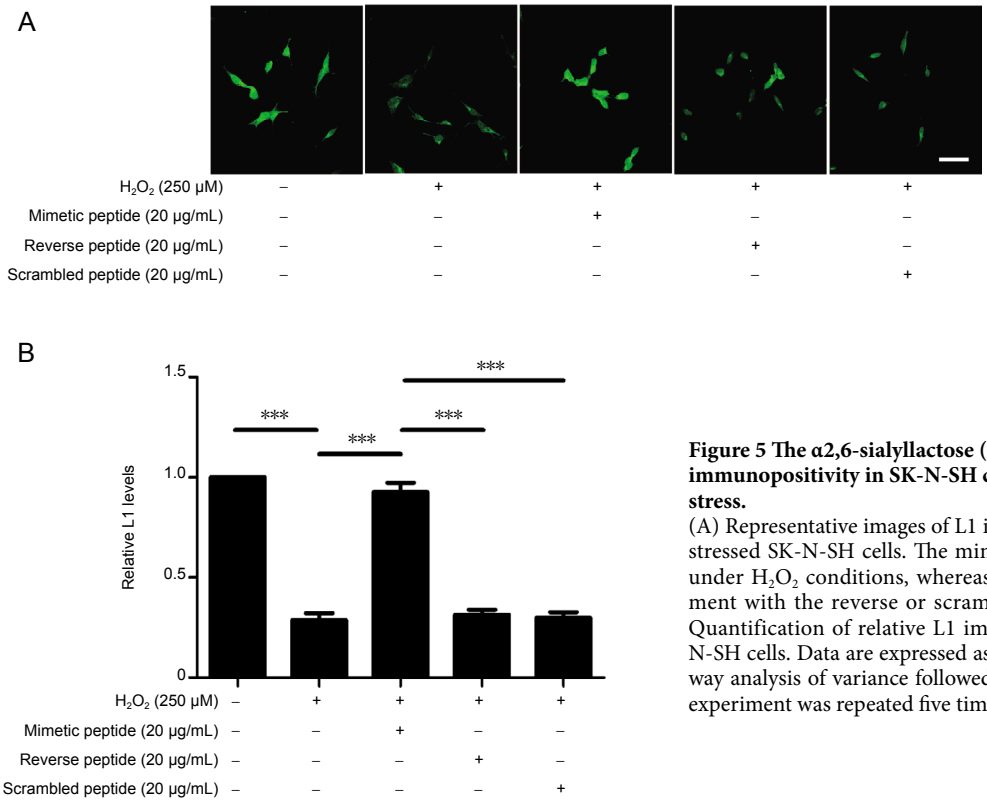


Figure 5 The α2,6-sialyllactose (6′-SL) mimetic peptide enhances L1 immunopositivity in SK-N-SH cells under H_2O_2-induced oxidative stress.

(A) Representative images of L1 immunofluorescence staining in H_2O_2-stressed SK-N-SH cells. The mimetic peptide increased L1 expression under H_2O_2 conditions, whereas L1 levels did not change after treatment with the reverse or scrambled peptides. Scale bar: 20 μm. (B) Quantification of relative L1 immunopositivity in H_2O_2-stressed SK-N-SH cells. Data are expressed as the mean ± SEM. $^{***}P < 0.001$ (one-way analysis of variance followed by the Bonferroni *post-hoc* test). The experiment was repeated five times.

cator for beneficial cell functions, because it plays a pivotal role in neural cell-to-cell interactions (Lindner et al., 1983; Faissner et al., 1984; Rathjen and Schachner, 1984) by supporting neuronal survival and migration, neurite outgrowth, axon guidance, myelination (Wood et al., 1990; Maness and Schachner, 2007; Sytnyk et al., 2017) and synaptic plasticity (Lüthi et al., 1994). Persistent expression of L1 is crucial for learning, memory, and regeneration following injury (Liljelund et al., 1994; Chaisuksunt et al., 2000; Zhang et al., 2005). L1 overexpression ameliorates the abnormal phe-

notypes in mouse models of neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease (Cui et al., 2010; Djogo et al., 2013). Under these auspices, we were able to show in the present study that the 6′-SL mimetic peptide increases L1 expression.

Taken together, our results reveal a neuroprotective role of the mimetic peptide for 6'-SL under oxidative stress conditions. Further studies are needed to identify the influence of the mimetic peptide in central nervous system injury, which are encouraged by the experience that adhesion molecules showing pro-activity *in vitro* can be pro-active *in vivo*. Also, it will be important to identify the downstream signaling molecules related to the neuroprotective effects of the mimetic peptide under pathological conditions with the hope that the mimetic peptide for α2,6-sialyllactose may be a novel candidate for therapy of neurodegenerative diseases. *In vivo* studies using animal models of neurodegenerative diseases may be needed to further identify the therapeutic efficacy of this peptide in the future. In conclusion, a mimetic peptide for 6′-SL with high affinity to Sambucus nigra lectin was identified by phage binding and competition ELISA. This peptide supports beneficial functions in cultured neurons, providing hopes for a peptide-based therapy to ameliorate the consequences of oxidative stress in neurodegenerative diseases in a clinical setting.

Author contributions: *Study conception, experiments design and discussion: WJZ, MS; experiments implementation: SXC, JHH, YJM, HFS, WJZ; data analysis and figures preparation: SXC, YJM; manuscript writing and revising: SXC, JHH, WJZ and MS. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*
Financial support: *This work was supported by the National Natural Science Foundation of China, No. 81471279 and No. 81171138 (to WJZ), Talent Support Grant from Shantou University Medical College, China, No. 2501220118 (to WJZ), and a grant from the Li Kashing Foundation, No. LD030302 (to MS). The funding bodies played no role in the study design, collection, analysis and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication.*
Institutional review board statement: *The study was approved by the Medical Ethics Committee of Shantou University Medical College, China (approval No. SUMC 2014-004) on February 20, 2014.*

Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*
Plagiarism check: *Checked twice by iThenticate.*
Peer review: *Externally peer reviewed.*

Open peer reviewers: *Roberta Cascella, University of Florence, Italy; Zhongfeng Shi, Guangdong Pharmaceutical University, China.*
Additional file: *Open peer review reports 1 and 2.*

References

Aghebati-Maleki L, Bakhshinejad B, Baradaran B, Motallebnezhad M, Aghebati-Maleki A, Nickho H, Yousefi M, Majidi J (2016) Phage display as a promising approach for vaccine development. J Biomed Sci 23:66.

Azzazy HM, Highsmith WE Jr (2002) Phage display technology: clinical applications and recent innovations. Clin Biochem 35:425-445.

Berns EJ, Alvarez Z, Goldberger JE, Boekhoven J, Kessler JA, Kuhn HG, Stupp SI (2016) A tenascin-C mimetic peptide amphiphile nanofiber gel promotes neurite outgrowth and cell migration of neurosphere-derived cells. Acta Biomater 37:50-58.

Burciu RG, Ofori E, Shukla P, Planetta PJ, Snyder AF, Li H, Hass CJ, Okun MS, McFarland NR, Vaillancourt DE (2015) Distinct patterns of brain activity in progressive supranuclear palsy and Parkinson's disease. Mov Disord 30:1248-1258.

Carri MT, Valle C, Bozzo F, Cozzolino M (2015) Oxidative stress and mitochondrial damage: importance in non-SOD1 ALS. Front Cell Neurosci 9:41.

Chaisuksunt V, Zhang Y, Anderson PN, Campbell G, Vaudano E, Schachner M, Lieberman AR (2000) Axonal regeneration from CNS neurons in the cerebellum and brainstem of adult rats: correlation with the patterns of expression and distribution of messenger RNAs for L1, CHL1, c-jun and growth-associated protein-43. Neuroscience 100:87-108.

Chang QY, Lin YW, Hsieh CL (2018) Acupuncture and neuroregeneration in ischemic stroke. Neural Regen Res 13:573-583.

Charbonneau MR, O'Donnell D, Blanton LV, Totten SM, Davis JC, Barratt MJ, Cheng J, Guruge J, Talcott M, Bain JR, Muehlbauer MJ, Ilkayeva O, Wu C, Struckmeyer T, Barile D, Mangani C, Jorgensen J, Fan YM, Maleta K, Dewey KG, et al. (2016) Sialylated milk oligosaccharides promote microbiota-dependent growth in models of infant undernutrition. Cell 164:859-871.

Chen X, Lan X, Mo S, Qin J, Li W, Liu P, Han Y, Pi R (2009) p38 and ERK, but not JNK, are involved in copper-induced apoptosis in cultured cerebellar granule neurons. Biochem Biophys Res Commun 379:944-948.

Colloby SJ, O'Brien JT, Taylor JP (2014) Patterns of cerebellar volume loss in dementia with Lewy bodies and Alzheimers disease: A VBM-DARTEL study. Psychiatry Res 223:187-191.

Cui YF, Hargus G, Xu JC, Schmid JS, Shen YQ, Glatzel M, Schachner M, Bernreuther C (2010) Embryonic stem cell-derived L1 overexpressing neural aggregates enhance recovery in Parkinsonian mice. Brain 133:189-204.

Djogo N, Jakovcevski I, Muller C, Lee HJ, Xu JC, Jakovcevski M, Kugler S, Loers G, Schachner M (2013) Adhesion molecule L1 binds to amyloid beta and reduces Alzheimer's disease pathology in mice. Neurobiol Dis 56:104-115.

Du J, Meledeo MA, Wang Z, Khanna HS, Paruchuri VD, Yarema KJ (2009) Metabolic glycoengineering: sialic acid and beyond. Glycobiology 19:1382-1401.

Ernst B, Magnani JL (2009) From carbohydrate leads to glycomimetic drugs. Nat Rev Drug Discov 8:661-677.

Faissner A, Kruse J, Nieke J, Schachner M (1984) Expression of neural cell adhesion molecule L1 during development, in neurological mutants and in the peripheral nervous system. Brain Res 317:69-82.

Farajdokht F, Amani M, Mirzaei Bavil F, Alihemmati A, Mohaddes G, Babri S (2017) Troxerutin protects hippocampal neurons against amyloid beta-induced oxidative stress and apoptosis. EXCLI J 16:1081-1089.

Fischer R, Maier O (2015) Interrelation of oxidative stress and inflammation in neurodegenerative disease: role of TNF. Oxid Med Cell Longev 2015:610813.

Galán A, Comor L, Horvatić A, Kuleš J, Guillemin N, Mrljak V, Bhide M (2016) Library-based display technologies: where do we stand? Mol Biosyst 12:2342-2358.

Ghoshal S, Bondada V, Saatman KE, Guttmann RP, Geddes JW (2016) Phage display for identification of serum biomarkers of traumatic brain injury. J Neurosci Methods 272:33-37.

Goldflam M, Ullman CG (2015) Recent advances toward the discovery of drug-like peptides de novo. Front Chem 3:69.

Hamzeh-Mivehroud M, Alizadeh AA, Morris MB, Church WB, Dastmalchi S (2013) Phage display as a technology delivering on the promise of peptide drug discovery. Drug Discov Today 18:1144-1157.

Hayashi-Takagi A, Araki Y, Nakamura M, Vollrath B, Duron SG, Yan Z, Kasai H, Huganir RL, Campbell DA, Sawa A (2014) PAKs inhibitors ameliorate schizophrenia-associated dendritic spine deterioration in vitro and in vivo during late adolescence. Proc Natl Acad Sci U S A 111:6461-6466.

Jeon J, Kang LJ, Lee KM, Cho C, Song EK, Kim W, Park TJ, Yang S (2018) 3′-Sialyllactose protects against osteoarthritic development by facilitating cartilage homeostasis. J Cell Mol Med 22:57-66.

Kamat PK, Kalani A, Rai S, Swarnkar S, Tota S, Nath C, Tyagi N (2016) Mechanism of oxidative stress and synapse dysfunction in the pathogenesis of Alzheimer's disease: understanding the therapeutics strategies. Mol Neurobiol 53:648-661.

Kang LJ, Kwon ES, Lee KM, Cho C, Lee JI, Ryu YB, Youm TH, Jeon J, Cho MR, Jeong SY, Lee SR, Kim W, Yang S (2018) 3′-Sialyllactose as an inhibitor of p65 phosphorylation ameliorates the progression of experimental rheumatoid arthritis. Br J Pharmacol 175:4295-4309.

Khodagholi F, Tusi SK, Alamdary SZ, Amini M, Ansari N (2012) 3-Thiomethyl-5,6-(dimethoxyphenyl)-1,2,4-triazine improves neurite outgrowth and modulates MAPK phosphorylation and HSPs expression in H_2O_2-exposed PC12 cells. Toxicol In Vitro 26:907-914.

Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. J Pharmacol Pharmacother 1:94-99.

Kraev I, Henneberger C, Rossetti C, Conboy L, Kohler LB, Fantin M, Jennings A, Venero C, Popov V, Rusakov D, Stewart MG, Bock E, Berezin V, Sandi C (2011) A peptide mimetic targeting trans-homophilic NCAM binding sites promotes spatial learning and neural plasticity in the hippocampus. PLoS One 6:e23433.

Ladeby R, Wirenfeldt M, Dalmau I, Gregersen R, Garcia-Ovejero D, Babcock A, Owens T, Finsen B (2005) Proliferating resident microglia express the stem cell antigen CD34 in response to acute neural injury. Glia 50:121-131.

Liljelund P, Ghosh P, van den Pol AN (1994) Expression of the neural axon adhesion molecule L1 in the developing and adult rat brain. J Biol Chem 269:32886-32895.

Lindner J, Rathjen FG, Schachner M (1983) L1 mono- and polyclonal antibodies modify cell migration in early postnatal mouse cerebellum. Nature 305:427-430.

Liu Y, Yu Y, Schachner M, Zhao W (2013) Neuregulin 1-beta regulates cell adhesion molecule L1 expression in the cortex and hippocampus of mice. Biochem Biophys Res Commun 441:7-12.

Liu YC, Yu MM, Chai YF, Shou ST (2017) Sialic acids in the immune response during sepsis. Front Immunol 8:1601.

Loers G, Liao Y, Hu C, Xue W, Shen H, Zhao W, Schachner M (2019) Identification and characterization of synthetic chondroitin-4-sulfate binding peptides in neuronal functions. Sci Rep 9:1064.

Lüthi A, Laurent JP, Figurov A, Muller D, Schachner M (1994) Hippocampal long-term potentiation and neural cell adhesion molecules L1 and NCAM. Nature 372:777-779.

Magnani JL, Ernst B (2009) Glycomimetic drugs--a new source of therapeutic opportunities. Discov Med 8:247-252.

Maness PF, Schachner M (2007) Neural recognition molecules of the immunoglobulin superfamily: signaling transducers of axon guidance and neuronal migration. Nat Neurosci 10:19-26.

Marino P, Norreel JC, Schachner M, Rougon G, Amoureux MC (2009) A polysialic acid mimetic peptide promotes functional recovery in a mouse model of spinal cord injury. Exp Neurol 219:163-174.

Martin-Sosa S, Martin MJ, Garcia-Pardo LA, Hueso P (2003) Sialyloligosaccharides in human and bovine milk and in infant formulas: variations with the progression of lactation. J Dairy Sci 86:52-59.

Masand SN, Chen J, Perron IJ, Hammerling BC, Loers G, Schachner M, Shreiber DI (2012) The effect of glycomimetic functionalized collagen on peripheral nerve repair. Biomaterials 33:8353-8362.

Mehanna A, Mishra B, Kurschat N, Schulze C, Bian S, Loers G, Irintchev A, Schachner M (2009) Polysialic acid glycomimetics promote myelination and functional recovery after peripheral nerve injury in mice. Brain 132:1449-1462.

Mehanna A, Jakovcevski I, Acar A, Xiao M, Loers G, Rougon G, Irintchev A, Schachner M (2010) Polysialic acid glycomimetic promotes functional recovery and plasticity after spinal cord injury in mice. Mol Ther 18:34-43.

Messing J (2016) Phage M13 for the treatment of Alzheimer and Parkinson disease. Gene 583:85-89.

Moldovan M, Pinchenko V, Dmytriyeva O, Pankratova S, Fugleholm K, Klingelhofer J, Bock E, Berezin V, Krarup C, Kiryushko D (2013) Peptide mimetic of the S100A4 protein modulates peripheral nerve regeneration and attenuates the progression of neuropathy in myelin protein P0 null mice. Mol Med 19:43-53.

Nagao M, Fujiwara Y, Matsubara T, Hoshino Y, Sato T, Miura Y (2017) Design of glycopolymers carrying sialyl oligosaccharides for controlling the interaction with the influenza virus. Biomacromolecules 18:4385-4392.

Owczarek S, Soroka V, Kiryushko D, Larsen MH, Yuan Q, Sandi C, Berezin V, Bock E (2011) Neuroplastin-65 and a mimetic peptide derived from its homophilic binding site modulate neuritogenesis and neuronal plasticity. J Neurochem 117:984-994.

Prost LR, Grim JC, Tonelli M, Kiessling LL (2012) Noncarbohydrate glycomimetics and glycoprotein surrogates as DC-SIGN antagonists and agonists. ACS Chem Biol 7:1603-1608.

Rathjen FG, Schachner M (1984) Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. EMBO J 3:1-10.

Ritchie CW, Bush AI, Mackinnon A, Macfarlane S, Mastwyk M, MacGregor L, Kiers L, Cherny R, Li QX, Tammer A, Carrington D, Mavros C, Volitakis I, Xilinas M, Ames D, Davis S, Beyreuther K, Tanzi RE, Masters CL (2003) Metal-protein attenuation with iodochlorhydroxyquin (clioquinol) targeting Abeta amyloid deposition and toxicity in Alzheimer disease: a pilot phase 2 clinical trial. Arch Neurol 60:1685-1691.

Ross RA, Spengler BA (2007) Human neuroblastoma stem cells. Semin Cancer Biol 17:241-247.

Ross RA, Biedler JL, Spengler BA (2003) A role for distinct cell types in determining malignancy in human neuroblastoma cell lines and tumors. Cancer Lett 197:35-39.

Simon-Haldi M, Mantei N, Franke J, Voshol H, Schachner M (2002) Identification of a peptide mimic of the L2/HNK-1 carbohydrate epitope. J Neurochem 83:1380-1388.

Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228:1315-1317.

Sytnyk V, Leshchyns'ka I, Schachner M (2017) Neural cell adhesion molecules of the immunoglobulin superfamily regulate synapse formation, maintenance, and function. Trends Neurosci 40:295-308.

Tan RH, Devenney E, Dobson-Stone C, Kwok JB, Hodges JR, Kiernan MC, Halliday GM, Hornberger M (2014) Cerebellar integrity in the amyotrophic lateral sclerosis-frontotemporal dementia continuum. PLoS One 9:e105632.

Tarr AJ, Galley JD, Fisher SE, Chichlowski M, Berg BM, Bailey MT (2015) The prebiotics 3′Sialyllactose and 6′Sialyllactose diminish stressor-induced anxiety-like behavior and colonic microbiota alterations: Evidence for effects on the gut-brain axis. Brain Behav Immun 50:166-177.

ten Bruggencate SJ, Bovee-Oudenhoven IM, Feitsma AL, van Hoffen E, Schoterman MH (2014) Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. Nutr Rev 72:377-389.

Torregrossa P, Buhl L, Bancila M, Durbec P, Schafer C, Schachner M, Rougon G (2004) Selection of poly-alpha 2,8-sialic acid mimotopes from a random phage peptide library and analysis of their bioactivity. J Biol Chem 279:30707-30714.

Varki A (2008) Sialic acids in human health and disease. Trends Mol Med 14:351-360.

Volpe M, Paneni F (2015) Shooting vascular oxidative stress: new hopes for stroke patients? Eur Heart J 36:1573-1575.

Wang B, Brand-Miller J (2003) The role and potential of sialic acid in human nutrition. Eur J Clin Nutr 57:1351-1369.

Wang H, Dharmalingam P, Vasquez V, Mitra J, Boldogh I, Rao KS, Kent TA, Mitra S, Hegde ML (2017) Chronic oxidative damage together with genome repair deficiency in the neurons is a double whammy for neurodegeneration: Is damage response signaling a potential therapeutic target? Mech Ageing Dev 161:163-176.

Wolf RC, Thomann PA, Sambataro F, Wolf ND, Vasic N, Landwehrmeyer GB, Sussmuth SD, Orth M (2015) Abnormal cerebellar volume and corticocerebellar dysfunction in early manifest Huntington's disease. J Neurol 262:859-869.

Wood PM, Schachner M, Bunge RP (1990) Inhibition of Schwann cell myelination in vitro by antibody to the L1 adhesion molecule. J Neurosci 10:3635-3645.

Xu J, Hu C, Chen S, Shen H, Jiang Q, Huang P, Zhao W (2017) Neuregulin-1 protects mouse cerebellum against oxidative stress and neuroinflammation. Brain Res 1670:32-43.

Zehra S, Khambati I, Vierhout M, Mian MF, Buck R, Forsythe P (2018) Human milk oligosaccharides attenuate antigen-antibody complex induced chemokine release from human intestinal epithelial cell lines. J Food Sci 83:499-508.

Zhang JX, Wang R, Xi J, Shen L, Zhu AY, Qi Q, Wang QY, Zhang LJ, Wang FC, Lu HZ, Hu JG (2017) Morroniside protects SK-N-SH human neuroblastoma cells against H_2O_2-induced damage. Int J Mol Med 39:603-612.

Zhang Y, Bo X, Schoepfer R, Holtmaat AJ, Verhaagen J, Emson PC, Lieberman AR, Anderson PN (2005) Growth-associated protein GAP-43 and L1 act synergistically to promote regenerative growth of Purkinje cell axons in vivo. Proc Natl Acad Sci U S A 102:14883-14888.

P-Reviewers: Cascella P, Shi Z; C-Editor: Zhao M; S-Editors: Yu J, Li CH; L-Editors: Yu J, Song LP; T-Editor: Jia Y