Molecular Neurobiology (2019) 56:7774–7788
https://doi.org/10.1007/s12035-019-1621-3

GALECTIN-8 Is a Neuroprotective Factor in the Brain that Can Be Neutralized by Human Autoantibodies

Evelyn Pardo[1] · Francisca Barake[1,2] · Juan A. Godoy[1] · Claudia Oyanadel[2] · Sofía Espinoza[1,2] · Claudia Metz[1,2] · Claudio Retamal[2] · Loreto Massardo[2] · Cheril Tapia-Rojas[2,3] · Nibaldo C. Inestrosa[1,4,5] · Andrea Soza[1,2] · Alfonso González[1,2,3]

Received: 18 February 2019 / Accepted: 23 April 2019 / Published online: 22 May 2019

Abstract

Galectin-8 (Gal-8) is a glycan-binding protein that modulates a variety of cellular processes interacting with cell surface glycoproteins. Neutralizing anti-Gal-8 antibodies that block Gal-8 functions have been described in autoimmune and inflammatory disorders, likely playing pathogenic roles. In the brain, Gal-8 is highly expressed in the choroid plexus and accordingly has been detected in human cerebrospinal fluid. It protects against central nervous system autoimmune damage through its immunesuppressive potential. Whether Gal-8 plays a direct role upon neurons remains unknown. Here, we show that Gal-8 protects hippocampal neurons in primary culture against damaging conditions such as nutrient deprivation, glutamate-induced excitotoxicity, hydrogen peroxide (H_2O_2)-induced oxidative stress, and β-amyloid oligomers (Aβo). This protective action is manifested even after 2 h of exposure to the harmful condition. Pull-down assays demonstrate binding of Gal-8 to selected β1-integrins, including α3 and α5β1. Furthermore, Gal-8 activates β1-integrins, ERK1/2, and PI3K/AKT signaling pathways that mediate neuroprotection. Hippocampal neurons in primary culture produce and secrete Gal-8, and their survival decreases upon incubation with human function-blocking Gal-8 autoantibodies obtained from lupus patients. Despite the low levels of Gal-8 expression detected by real-time PCR in hippocampus, compared with other brain regions, the complete lack of Gal-8 in Gal-8 KO mice determines higher levels of apoptosis upon H_2O_2 stereotaxic injection in this region. Therefore, endogenous Gal-8 likely contributes to generate a neuroprotective environment in the brain, which might be eventually counteracted by human function-blocking autoantibodies.

Keywords Galectins · Neuroprotection · Integrins · ERK1/2 · PI3K/AKT · Hippocampal neurons

✉ Andrea Soza
andrea.soza@uss.cl

✉ Alfonso González
agonzara@uss.cl

1 Centro de Envejecimiento y Regeneración (CARE), Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

2 Centro de Biología Celular y Biomedicina (CEBICEM), Facultad de Medicina y Ciencia, Universidad San Sebastián, Santiago, Chile

3 Fundación Ciencia y Vida, Santiago, Chile

4 Center for Healthy Brain Ageing, University of New South Wales, Sydney, NSW, Australia

5 Center of Excellence in Biomedicine of Magallanes (CEBIMA), Universidad de Magallanes, Punta Arenas, Chile

Introduction

Neurons in the brain are exposed to many harmful conditions that can lead to cell death by activating apoptotic pathways [1–3]. Abnormal neuronal death characteristically occurs in neurological diseases such as stroke, Alzheimer's disease (AD), and multiple sclerosis (MS) [3]. Also in systemic lupus erythematosus (SLE), the prototypic autoimmune disease, neuronal death likely contributes to developing neuropsychiatric SLE (NPSLE) associated with a variety of neurotoxic factors, including brain-reactive autoantibodies [4–7]. The mechanisms that balance neuronal survival versus death pathways in the brain remain incompletely understood and therefore the therapeutic alternatives for these diseases are currently limited [1–4]. A decreased function of neuroprotective factors in the brain might be one of the conditions contributing to

neurodegeneration and neuropsychiatric disorders in different brain diseases [8, 9]. On the other hand, harmful elements such as excitotoxicity due to an exaggerated release of glutamate and oxidative stress by increased oxygen reactive species are common to acute ischemia and chronic neurodegenerative diseases [2, 3, 10], while β-amyloid (Aβ) contributes to overpass the natural neuroprotective systems particularly in AD [9]. Indeed, identifying new endogenous factors that promote neuroprotection against apoptosis-inducers such as glutamate, hydrogen peroxide (H_2O_2) and Aβ-oligomers (Aβo) might reveal new clues regarding the neural survival/death balance and how to improve current treatments.

Galectins constitute a family of 15 proteins that share homologous carbohydrate-recognition domains (CRDs) for β-galactosides and display a wide spectrum of functions acting as glycan-based decoders, both within the cytosolic compartment and the extracellular space [11–13]. In the cytosol, galectins can play a variety of functions. They can regulate gene transcription and mRNA splicing through nonglycan interactions [14], while their glycan interactions can mediate recognition and autophagic removal of damaged endomembranes, thus having a protective role at least in innate immunity [11, 15]. After unconventional secretion, presumably involving exosomes [16], galectins interact at the cell surface with a variety of glycosylated molecules, including signaling receptors, and can either establish or interfere with cell-to-cell and cell-to-extracellular matrix (ECM) interactions, modifying cellular processes with physiologic and therapeutic implications [13, 17]. Galectin-1 (Gal-1), Gal-3, Gal-4, Gal-8, and Gal-9 seem to be significantly expressed in the brain [18]. However, most studies on neuronal function and protection have been focused on Gal-1 and Gal-3 [14].

We have been studying Gal-8 [19], which belongs to the tandem-repeat class of galectins characterized by two CRDs bound by a linker peptide of variable length. Gal-8 is widely expressed in many tissues and tumors and has a unique specificity for α2,3-sialylated glycans in its N-terminal CRD, not shared by other galectins, likely entailing special functional properties [20]. Intracellularly, Gal-8 promotes autophagic removal of damaged endosomes and lysosomes, linking their cytosolic exposed luminal glycans to the NDP52 autophagy adaptor [15] and inhibiting mTOR in the lysosomal membrane [21]. This cell-protecting system constitutes a defense mechanism against bacterial [15, 21] and viral invasion [22] and might also protect against endocytic-dependent propagation of the pathogenic tau protein relevant in AD [23]. The role of Gal-8 in the extracellular space is exerted through glycan-dependent interactions with selected cell surface signaling receptors and integrins [24, 25]. Gal-8 binds and activates selected β1-integrins in different cellular systems [26–28], which can trigger ERK1/2 and PI3K/AKT signaling pathways [26, 28–30]. β1-Integrins and ERK1/2 and PI3K/AKT survival signaling pathways are important components of the neuroprotection machinery [2, 31–36]. We recently described Gal-8 expression in several regions of the mouse brain, with high levels in the choroid plexus that generates cerebrospinal fluid (CSF), and accordingly, we detected Gal-8 in human CSF [19]. However, the role of Gal-8 as neuroprotector has not been explored.

An additional feature to consider regarding the roles of Gal-8 is that human anti-Gal-8 autoantibodies seems to be frequent in autoimmune or chronic inflammatory disorders, including rheumatoid arthritis, SLE, and MS [19, 37]. These antibodies can neutralize Gal-8 interactions with cell surface glycoproteins, including β1-integrins, and their functional outcomes [28, 30, 37]. Other antibodies have been shown to interfere with neuronal function and can lead to neuronal death in the brain, associating with NPSLE [5, 7]. Our previous studies on experimental autoimmune encephalomyelitis and patients with remitting-recurrent MS (RRMS) revealed that Gal-8 protects against CNS inflammatory damage acting as immune-suppressor [19]. This Gal-8 function is counteracted by function-blocking anti-Gal-8 antibodies generated by RRMS patients, with pathogenic consequences reflected in a worse prognosis due to neuronal damage [19]. Anti-Gal-8 antibodies can be found in the CSF of MS patients [19].

All this data prompts to define not only the role of Gal-8 on neuronal responses to injuring conditions relevant to acute and chronic neurological disorders but also the effect of human function-blocking anti-Gal-8 antibodies upon neuronal survival.

Materials and Methods

Antibodies and Reagents

Polyclonal antibodies against integrins α2, α3, α4, α5, and β1, and mouse blocking anti-β1 integrins were purchased from Chemicon International, Inc., CA. Anti-ERK and anti-pERK were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-AKT and anti-pAKT (Ser473) from Cell Signaling Technology. Anti-caspase-3, anti-caspase-9, and anti-cytochrome *c*, were from Santa Cruz Biotechnology (Santa Cruz, CA). Thiodigalactoside (TDG), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342, thrombin, wortmannin, protein-A-Sepharose, and glutamate were from Sigma Aldrich CO, St. Louis USA. Enhanced chemiluminescence (ECL) system was from Amersham (Piscataway, NJ). PD98059 was from Calbiochem (La Jolla, CA) and hydrogen peroxide (H_2O_2) from Merck (Darmstadt, Germany). Anti-Gal-8 (anti-rGal-8) antibodies obtained by immunizing rabbits with recombinant human Gal-8 were previously characterized [28, 30].

Isolation of Human Function-Blocking Anti-Gal-8 Autoantibodies and Anti-Gal-8-Depleted IgG Fraction

Thirty patients with SLE attending the outpatient clinic of the Department of Clinical Immunology and Rheumatology, Faculty of Medicine, Pontificia Universidad Católica de Chile, donated sera in agreement with an informed consent approved by the ethical committee of the same institution. Sera were screened by immunoblot for autoantibodies against Gal-8 using 0.5 μg of Gal-8 released from glutathione-*S*-transferase (GST)-Gal-8 as an antigen, as previously described [37]. Sera that gave strong reactivity were used to purify Gal-8 autoantibodies by affinity chromatography with recombinant Gal-8 covalently linked to Affi-Gel 10 (BioRad), according to the manufacturer's instructions. Bound antibodies were eluted with 0.1 M glycine-HCl at pH 2.5 solution, neutralized with 1 M K_2HPO_4, dialyzed in PBS, and maintained in PBS containing 1 mg ml^{-1} BSA at − 20 °C. The remaining anti-Gal-8-depleted sera were incubated with protein-A-Sepharose to obtain a control IgG fraction (C-IgG). Human anti-Gal-8 antibodies can be used as function-blocking antibodies [19, 37].

Recombinant Gal-8 Production

Transformed *Escherichia coli* were incubated with 0.2 mM isopropyl-1-thio-h-d-galactopyranoside (Invitrogen) for 4 h to induce the expression of GST-Gal-8 protein and purified by affinity chromatography with glutathione-Sepharose as described [28, 38]. Gal-8 was released from GST-Gal-8 linked to glutathione-Sepharose by thrombin treatment (10 U mg^{-1} of fusion protein) for 4 h at room temperature.

Hippocampal Neuron Primary Cultures

Hippocampal primary cultures were prepared from E18 Sprague-Dawley rats, treated with 2 μM cytosine arabinoside for 24 h to decrease glial growth, and maintained in B27-supplemented mneurobasal media, as previously described [39, 40]. Experiments were performed on 8–10 days in vitro (DIV) cultures.

Cell Viability Assay

Hippocampal neurons (2×10^4 cells 100 ml^{-1} $well^{-1}$) were incubated for 24 h at 37 °C under different conditions and then cell viability was measured by the MTT reduction method, as previously described [41, 42].

Aβ Oligomers Preparation

Synthetic Aβ1–42 peptide corresponding to wild-type human Aβ was obtained from Genemed Synthesis, Inc. (San Francisco, CA) and used to prepare Aβo, as described [43, 44]. Briefly, Aβ peptide stock solution was prepared by dissolving freeze-dried aliquots of Aβ in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma H-8508) at 1 mM, incubated at room temperature for 1 h and lyophilized. For Aβo preparation, peptide film was dissolved in dimethyl sulfoxide (DMSO, Sigma D2650) at 5 mM and then diluted into distilled water to a final concentration of 100 μM. The preparation was incubated overnight for Aβo formation.

Immunofluorescence

Hippocampal neurons plated on polylysine-coated coverslips (30,000 neurons/cover) were softly rinsed twice in ice-cold PBS, fixed with freshly prepared 4% paraformaldehyde in PBS for 20 min, and permeabilized for 5 min with 0.2% Triton X-100 in PBS. After several washes in ice-cold PBS, cells were incubated in 1% BSA in PBS (blocking solution) for 30 min at room temperature, followed by an overnight incubation at 4 °C with the indicated primary antibodies, extensively washed with PBS, and then incubated with Alexa-conjugated secondary antibodies for 30 min at 37 °C. Digital images were acquired on a Zeiss Axiophot microscope using the × 63 immersion objective and a 14-bit Axiocam camera and a Axiovision imaging software [45]. For quantification, neurites were segmented in ROIs of 2 μm, and the average intensity of each ROI was measured and normalized respect to control.

Integrin Binding to Gal-8

Pull-down experiments to assess integrin binding to Gal-8 were performed as described [28]. Briefly, hippocampal cells (1×10^6 cells) were lysed in 1% NP-40 buffer containing 25 mM Tris/HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 4 μg ml^{-1} leupeptin, 4 mM PMSF, and 4 μg ml^{-1} pepstatin A (Sigma) for 1 h at 4 °C. The lysates were cleared by centrifugation at 4 °C for 10 min at 10,000× *g*, and the supernatants were first incubated with GST attached to glutathione-Sepharose for 1 h at 4 °C and then with 25 μl of GST-Gal-8 linked to glutathione-Sepharose beads. To assess glycan-dependent Gal-8 interactions, competence experiments were performed by preincubating GST-Gal-8 beads with 10 mM TDG for 1 h at 37 °C. The beads were then washed in PBS, suspended, and boiled in loading buffer. Proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and incubated with the indicated antibodies against α and β integrin subunits and then visualized by ECL.

Gal-8 Detection in Cell Extracts and Conditioned Media

To analyze secretion of Gal-8, hippocampal neurons were incubated with Neurobasal/B27 media, supplemented with 100 μCi ml^{-1} of [^{35}S] methionine/cysteine for 16 h. Then,

conditioned media was collected, centrifuged at 20,000×*g* for 10 min, and Gal-8 was immunoprecipitated with α-hGal-8 or pulled down with lactose-coated beads [46].

Animals

Sprague-Dawley rats and mice were housed at the Faculty of Biological Sciences of the Pontificia Universidad Católica de Chile and handled according to guidelines outlined and approved by the Institutional Animal Care and Use Committee of this Faculty. Animals were euthanized by an overdose of anesthesia. *Lgals8*/Lac-Z knock-in (here called Gal-8 KO or *Lgals8*$^{-/-}$) mice [19] were generated from *C57BL/6NTac* mice engineered in Regeneron Pharmaceuticals Inc., New York, using Velocigene technology [47] for replacing the entire coding region of the mouse *Lgals8* gene (18,427 bp) with LacZ lox-Ub1-EM7-Neo-lox Cassette, containing the LacZ gene that encodes β-galactosidase. Details of the *Lgals8* KO mice and PCR genotyping assay, including the predicted PCR products and the primers, are available at the Velocigene website (www.velocigene.com/komp/detail/14305).

CNS β-gal histochemistry and histological analysis

Brains were fixed by perfusion in 4% paraformaldehyde (PFA) in PB buffer (0.1 M phosphate buffer at pH 7.4) and processed for β-gal histochemistry as described [48, 49].

Gal-8 mRNA Detection by Real-Time PCR

Total mRNA was isolated from mouse brain by using Trizol reagent according to the manufacturer protocol. Real-time PCR was performed using the SYBR Green Master Mix Kit. Primer sequences were: β-actin forward GATGACCCAGATCA TGTTTG, β-actin reverse CTTCTCTTTGATGTCACGCA, Gal-8 forward TCCATCGGGTTCAGATTCAG, Gal-8 reverse GCACACCATTTATAGCAACC. Thermocycler conditions included an initial hold at 95 °C for 5 min followed by a two-step PCR program at 95 °C for 5 s and 60 °C for 10 s, repeated for 40 cycles in a Rotor-Gene Q System (QIAGEN). The amount of endogenous β-actin mRNA was used for an internal control for qPCR in each sample.

Stereotaxic Injection

Animals received bilateral intrahippocampal administration of H_2O_2 by stereotaxic injection as previously described [50]. Gal8 KO and wild-type (WT) mice were anesthetized using 1.5–2.5% isoflurane. The head was shaved and the animal was placed in a small, mouse stereotaxic frame (Stoelting) with nontraumatic ear bars to hold the skull in place. The skull was exposed from several millimeters anterior and posterior to bregma. Bore holes were made above the left and right CA1 hippocampus (coordinates, 2.46 mm anterior to the bregma, 1.0 mm lateral, 1.5 mm relative to dura mater). Body temperature during anesthesia was maintained at 37 °C by an isothermal heating pad. Mice were allowed to recover for 48 h before euthanasia. Sterile H_2O_2 was diluted in PBS 1× to reach a final concentration of 50 μM, previously reported to promote cell death [51, 52].

Immunoblot

To assess ERK1/2 and AKT activation, proteins (25 μg) of cell lysates prepared from 1×10^6 cells in the presence of phosphatase inhibitors were resolved in 10% SDS-PAGE, transferred to nitrocellulose membranes, and analyzed with antibodies against phosphorylated ERK1/2 or AKT at 1:1000 dilution. The bands were digitalized and their relative intensities estimated by NIH J software. To analyze the apoptotic effect of H_2O_2 stereotaxic injection, the hippocampus of WT and Gal-8 KO mice were dissected on ice and immediately processed as previously described [9, 53]. Briefly, hippocampal tissue was homogenized in RIPA buffer (10 mM Tris-Cl, pH 7.4, 5 mM EDTA, 1% NP-40, 1% sodium deoxycholate, and 1% SDS) supplemented with a protease inhibitor mixture and phosphatase inhibitors (25 mM NaF, 100 mM Na3VO4, and 30 μM $Na_4P_2O_7$) using a Potter homogenizer and then sequentially passed through syringes of different calibers. Protein samples were centrifuged twice at 14,000 rpm for 15 min at 4 °C, and the concentrations were determined using the BCA Protein Assay Kit (Pierce). The samples were resolved by SDS-PAGE, followed by immunoblotting on PVDF membranes with the corresponding antibodies.

Statistical Analysis

Experiments were made in replicates ($n = 3$, except when indicated). Data are expressed as mean ± standard error. Apparent differences were assessed for statistical significance using GraphPad Prism and Sigma Plot software. Significance was accepted at p value < 0.05. p values correspond to $^*p < 0.05$ and $^{**}p < 0.001$.

Results

Gal-8 Protects Neurons Against Damaging Conditions

To study the role of Gal-8 on neuronal viability, we challenged hippocampal neurons in primary culture with well-known neuronal damaging conditions. Nutrient deprivation is a strong stressor that decreases the viability of neurons in primary culture [54]. Incubation of 8–10 DIV cultures of hippocampal neurons for 24 h in the absence of B27 supplement showed decreased survival, which could be counteracted by

adding Gal-8 (1.5 μM) to the media (Fig. 1a; $*p < 0.01$). To determine whether this protective effect involves interactions of Gal-8 with cell surface glycans, we used TDG that blocks galectin/glycan-interactions [28, 30]. Pre-incubation of Gal-8 with TDG inhibited the survival effect of Gal-8 (Fig. 1a). Therefore, Gal-8 interactions with cell surface glycans promote survival of hippocampal neurons challenged by nutrient withdrawal.

We then studied whether Gal-8 protects neurons against injuring conditions such as glutamate-induced excitotoxicity and H_2O_2-mediated oxidative stress, as common agents in many neurological diseases, and Aβo particular to AD pathogenesis [55–57]. We tested the effect of Gal-8 in the absence of B27 to further stress the neuronal survival system. Hippocampal neurons incubated without B27 showed even lower levels of viability under glutamate (15 μM) (Fig. 1b; $*p < 0.05$), H_2O_2 (25 μM) (Fig. 1c; $**p < 0.001$) and Aβ-peptide (5 μM) (Fig. 1d; $**p < 0.001$) incubation. In all these conditions, co-incubation with 1.5 μM of Gal-8 improved survival in a TDG blockable manner (Fig. 1a–d). Gal-8 treatment also decreased the rate of apoptosis induced by both glutamate and H_2O_2, as demonstrated by quantifying Hoechst-stained pyknotic nuclei in neurons identified with anti-MAP2B antibodies (Fig. 2). Therefore, Gal-8 also protects against apoptotic stimuli conveyed by excitotoxicity and oxidative stress in hippocampal neurons.

An important aspect to evaluate in neuroprotective factors is their potential to rescue cells previously exposed to cell injuring conditions. We pre-treated cells with glutamate for different time periods before adding Gal-8 (1.5 μM) and assessed neuronal survival after 22 h. Strikingly, Gal-8 precluded death of neurons previously exposed for up to 2 h to glutamate (15 μM) treatment ($p < *0.01$) (Fig. 3). After this period, Gal-8 is no longer protected. Although some

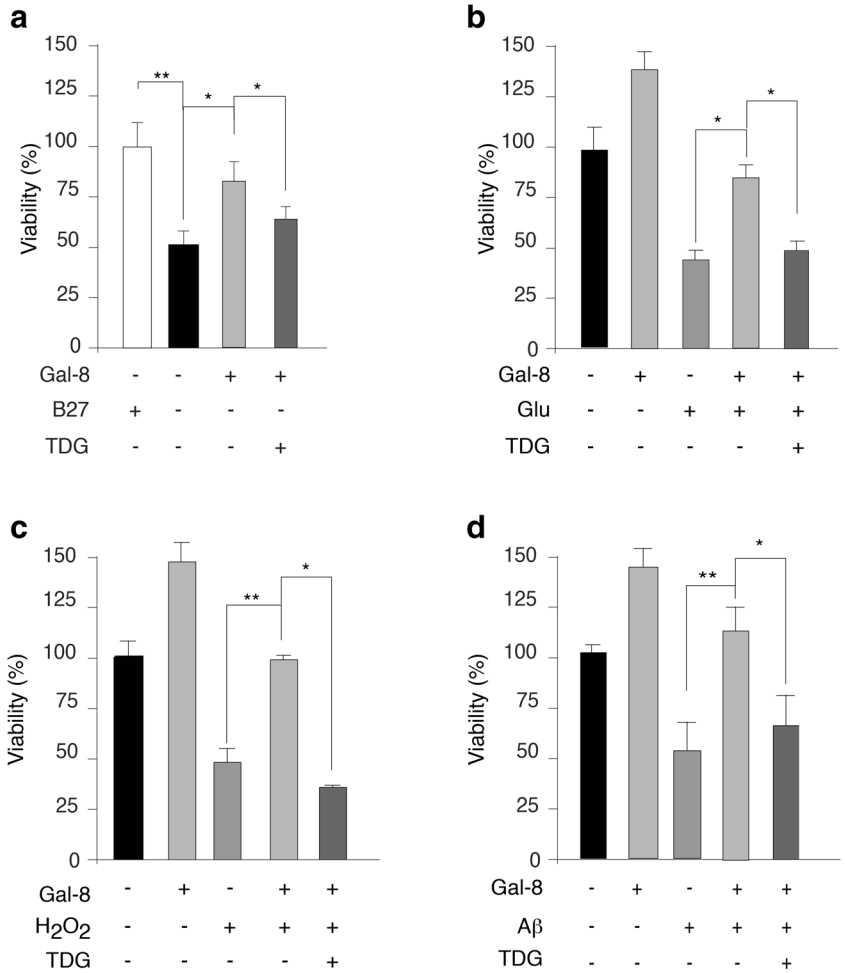

Fig. 1 Gal-8 improves neuronal survival and protects against damaging conditions. Primary hippocampal neurons were incubated for 24 h at 37 °C in neurobasal medium with or without B27 supplement and absence or presence of 1.5 μM Gal-8 and 20 mM TDG, as indicated. Cell viability was assessed by MTT. **a** Nutrient and growth factor deprivation by omitting B27 supplement decreased cell viability by 50% in the absence of Gal-8 versus 25% in the presence of Gal-8. In the absence of B27, Gal-8 counteracted the viability impairment caused by: **b** glutamate (15 μM), **c** H_2O_2 (25 μM), and **d** Aβo (5 μM). In all these harmful conditions, TDG prevented the neuroprotective effect of Gal-8, indicating dependency on lectin interactions with cell surface glycans. ($*p < 0.05$; $**p < 0.001$, ANOVA; $n = 3$, each of the three experiments in triplicate)

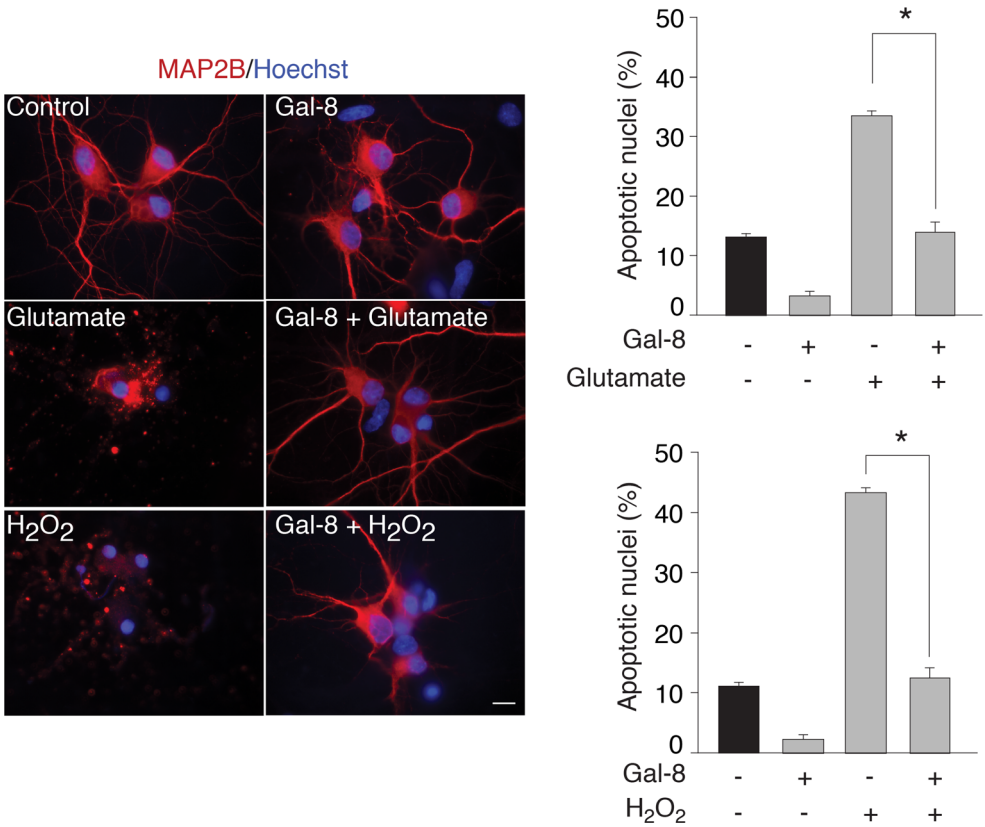

Fig. 2 Gal-8 prevents apoptosis in hippocampal neurons. Hippocampal neurons were incubated for 24 h at 37 °C in the absence or presence of glutamate (15 μM) or H_2O_2 (25 μM), with or without Gal-8 (1.5 μM), as indicated. Indirect immunofluorescence for MAP2B and the quantification of nuclei stained with Hoechst showed that Gal-8 lowered the percentage of apoptotic nuclei in neurons under both deleterious conditions (bar, 10 μm). (*$p < 0.01$ ANOVA; $n = 3$, each of the three experiments in triplicate, analyzing 60–80 cells per condition)

variability can be observed in the survival rates between experiments, likely due to intrinsic differences in the hippocampal primary neuronal culture, the conclusion of Gal-8 as a neuroprotective factor does not change as we always included internal controls in each experiment.

Gal-8-Mediated Neuroprotection Involves Activating Interactions with β1-Integrins

β1-Integrins, particularly α5β1 integrin [34], can account for most of ECM-dependent neuroprotection [31–33]. Gal-8 has been reported to bind selective β1-integrins, including α5β1 integrins, in other cellular systems [28, 30, 46]. Therefore, we analyzed the selectivity of Gal-8 for β1-integrins expressed in hippocampal neurons. We used recombinant GST-Gal-8 linked to glutathione-Sepharose to pull down glycoproteins and analyzed bound integrins by immunoblot against α2, α3, α4, α5, and β1 integrin subunits. As in other cells, Gal-8 preferentially bound α3 and α5 but not α2 or α4 integrins (Fig. 4a, lane 2). Indeed, we detected α2 and α4 subunits in the cell extracts (Fig. 4a, input). Therefore, the lack of α2 or α4 subunits in the pull down means that Gal-8 neither interacts with these subunits nor with the β1 subunit in the α2β1 or α4β1 dimeric integrin. Again, TDG inhibited the binding of Gal-8 to α-integrins (Fig. 4a, lane 3). These results indicate that among several β1-integrins expressed by neuronal cells Gal-8 interacts with β-galactosides present in selected α subunits, including α5.

Next, we studied whether Gal-8 activates β1-integrins with neuropotential consequences in hippocampal primary neurons. Immunofluorescence with an antibody that detects the active conformation of β1-integrin [34, 58] revealed that Gal-8 effectively activates β1-integrins in hippocampal neurons (Fig. 4b). A function-blocking antibody to β1-integrin blocked the β1-integrin activation induced by Gal-8 (Fig. 4b), thus giving the opportunity to explore the role of β1-integrin-activation in the neuroprotective effects of Gal-8. Hippocampal neurons pre-treated with the β1-integrin function-blocking antibodies no longer showed the survival and protective effects of Gal-8 tested against glutamate cytotoxicity (Fig. 4c). Taken together, these results indicate that Gal-8 protects neurons from harmful conditions by activating β1-integrins through its interactions with selective α-subunits, including α5 subunit.

Gal-8 Activates ERK and AKT Signaling Pathways that Contribute to Neuroprotection

Down-stream mediators of β1-integrin-driven cell survival and neuroprotection in response to ECM components include MAPK ERK1/2 and PI3K/AKT signaling pathways [31–34]. Treatment of primary neurons with Gal-8 showed activation of both ERK1/2 (sevenfold) and AKT (fivefold) over basal

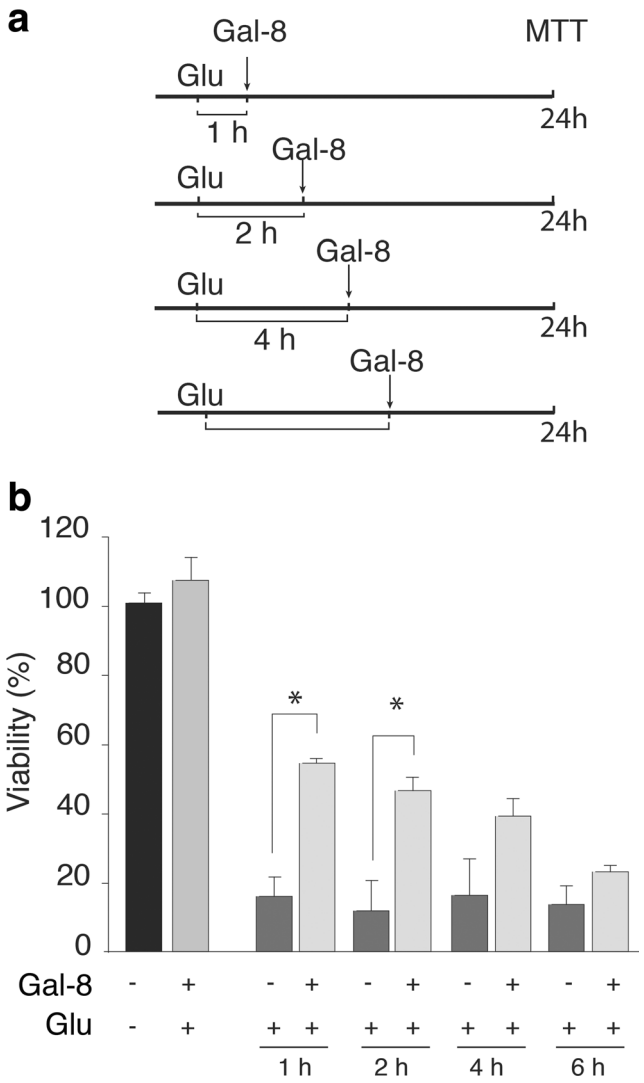

Fig. 3 Gal-8 protects neurons even 2 h after glutamate addition. Hippocampal neurons were incubated with glutamate (15 μM) for 1, 2, 4, and 6 h, and afterwards, Gal-8 (1.5 μM) was added for 22 h. Cell viability assessed by MTT assay showed improved survival when Gal-8 was added within 2 h after starting the glutamate challenge, losing its protective effect at a longer time point. (*$p < 0.01$ ANOVA; $n = 3$, each of the three experiments in triplicate)

levels (Fig. 5a). This activation decreased in the presence of TDG and thus involved Gal-8 interactions with cell surface glycans (Fig. 5a, lane 5). Inhibitors of ERK1/2 (25 μM PD98059) and PI3K/AKT (50 nM wortmannin) pathways decreased the neuroprotective effect of Gal-8 (Fig. 5b; *$p < 0.05$). Therefore, activation of both ERK1/2 and PI3K/AKT signaling pathways is involved in Gal-8-mediated neuroprotection.

Neurons Secrete Gal-8 Creating a Protective Environment that Can Be Blocked by Human Anti-Gal-8 Autoantibodies

Our experiments so far demonstrate neuroprotection using recombinant Gal-8 preparations. Galectins are secreted by some yet unknown unconventional mechanism likely including exosomes [16]. To define whether endogenously produced Gal-8 has similar effects, we assessed the production and secretion of Gal-8 in the hippocampal primary cultures. We metabolically labeled the cells with [^{35}S] methionine/cysteine and then performed immunoprecipitation or affinity chromatography with anti-hGal-8 autoantibody or with lactose-agarose, respectively. Both approaches detected a metabolically labeled 34 kDa protein corresponding to the molecular mass of Gal-8 (Fig. 6a). These results suggest that primary hippocampal neurons in primary culture express and secrete Gal-8.

We previously described that patients with SLE or with MS generate anti-Gal8 autoantibodies that block the interaction of Gal-8 with cell surface integrins, blocking its functional outcomes [19, 24, 28, 30, 59]. These function-blocking human anti-Gal-8 autoantibodies can be used to assess the contribution of endogenously secreted Gal-8 upon neuronal survival. Incubation with affinity-purified Gal-8 antibodies (0.5 μg ml^{-1}) from SLE patients decreased the viability of primary hippocampal neurons (Fig. 6b). In contrast, anti-hGal-8 serum pre-incubated with Gal-8 or depleted from anti-Gal-8 antibodies by affinity chromatography showed no effect (Fig. 6b). Therefore, the results suggesting that primary hippocampal neurons express and secrete Gal-8, and the decreased viability of these neurons upon incubation with function-blocking human anti-Gal-8 antibodies, indeed indicate that endogenously secreted Gal-8 contributes to neuronal survival. Importantly, at the same time these results demonstrate that human autoantibodies can neutralize the neuroprotective role of Gal-8.

Gal-8 Expression and Neuroprotection in Adult Brain

Our previous results indicate that fetal hippocampal neurons used for primary culture produce and secrete detectable and neuroprotective-competent levels of Gal-8. The question then is what might happen in the adult brain. We previously assessed the expression of Gal-8 in the adult mice brain using Gal-8 knock-in mice, which bear an insertion of β-galactosidase under the Gal-8 promoter, and thus β-galactosidase staining reflects Gal-8 expression [19]. As previously shown by Pardo et al. [19], β-galactosidase staining revealed higher Gal-8 expression levels in the choroid plexus and thalamus, whereas the hippocampus and cortex displayed relatively low levels of Gal-8 expression and mainly scattered to a few cells (Fig. 7a). A complementary analysis using real time PCR corroborated the higher levels of Gal-8 expression at the choroid plexus and at a brain segment encompassing the thalamus, hypothalamus, and midbrain, contrasting with detectable but low levels of expression at the hippocampus (Fig. 7b).

Finally, to test whether Gal-8 contributes to neuroprotection in the adult brain we compared the levels of apoptotic markers in

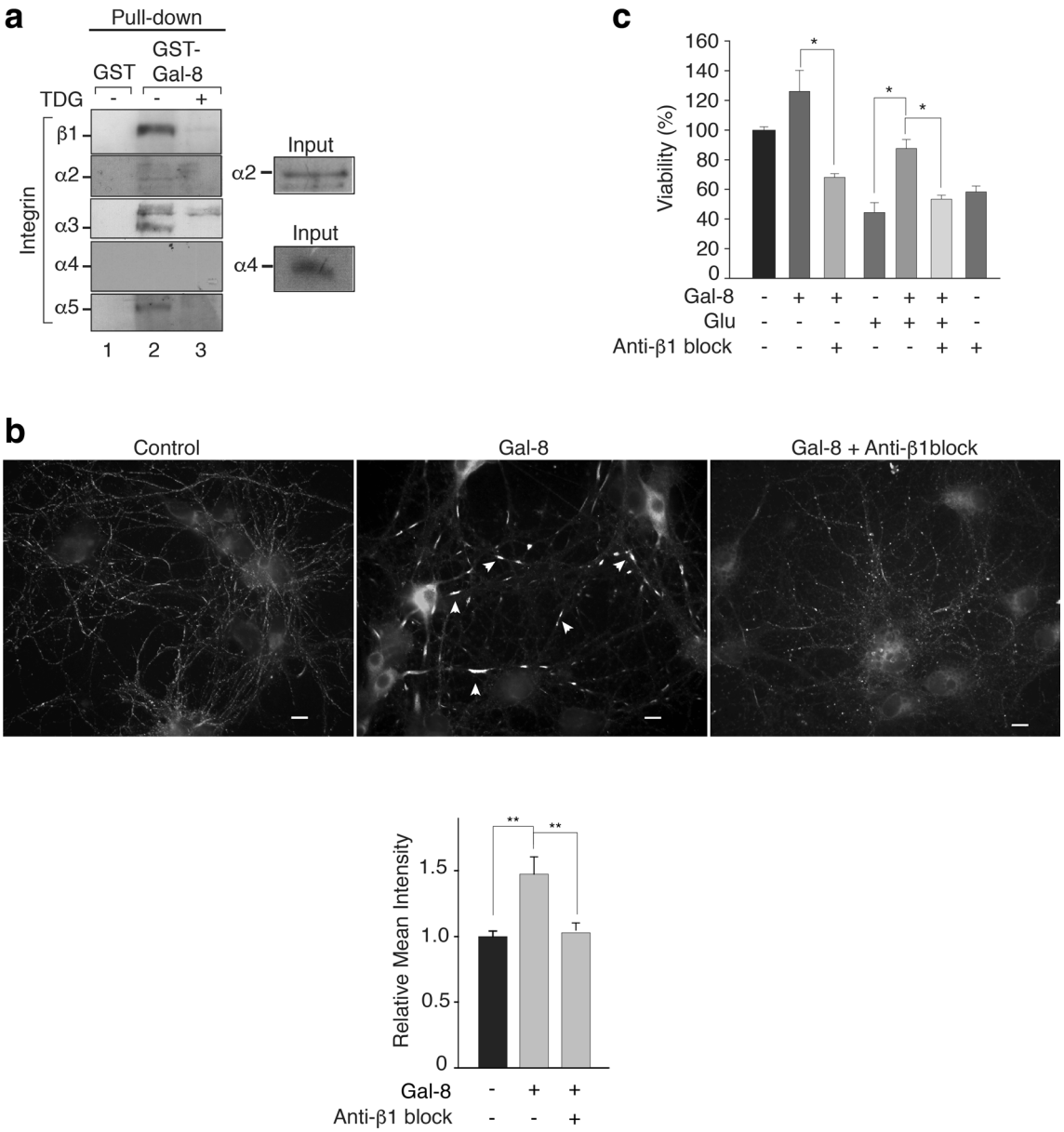

Fig. 4 β1-integrin activation mediates Gal-8 neuroprotection. **a** Pull down of hippocampal neuron glycoproteins with GST-Gal-8 linked to a glutathione-sepharose column and in the absence or presence of 20 mM TDG resolved by SDS-PAGE and immunobloted against the indicated integrin subunits. Gal-8 selectively bound to α3, α5, and β1 but not to α2 and α4 integrins, even though these subunits are detected in the whole cell extract. Gal-8 binding to selected α subunits involved interaction with β-galactosides, as indicated by TDG block. **b** Indirect immunofluorescence with an antibody that detects activated forms of β1-integrins showed positive signals (arrow heads) upon incubation of the cells with Gal-8 1.5 μM for 1 h. Pre-incubation with 20 μg ml^{-1} of anti-β1-blocking antibodies (anti-β1 block) prevented the Gal-8-induced β1-integrin activation. (bar, 10 μm). Graph below shows increased fluorescence intensity measured on neurites. (**$p < 0.001$ ANOVA; Relative mean intensity ± SEM in 30 ROIs from two experiments); c. Pre-incubation with anti-integrin β1 blocking antibodies (20 μg ml^{-1}) for 1 h also abrogated the Gal-8-mediated (1.5 μM) neuronal survival improvement against glutamate. (*$p < 0.01$ ANOVA; $n = 3$, each of the three experiments in triplicate)

the hippocampus of wild-type and Gal-8 KO mice after stereotaxic injection of H_2O_2. Immunoblot analysis of the hippocampus showed higher levels of cytochrome *c*, caspase-9 and caspase-3 in Gal-8 KO mice, reflecting an increased apoptotic damage, compared with WT mice (Fig. 8). These results demonstrate that endogenous Gal-8 expression contributes to neuroprotection counteracting apoptotic pathways in the brain.

Discussion

Our previous study reported Gal-8 in the brain and CSF playing an immunosupressive role that protects the CNS against autoimmune inflammatory conditions such as EAE and MS [19]. We now provide evidence that Gal-8 can directly protect neurons against harmful conditions commonly

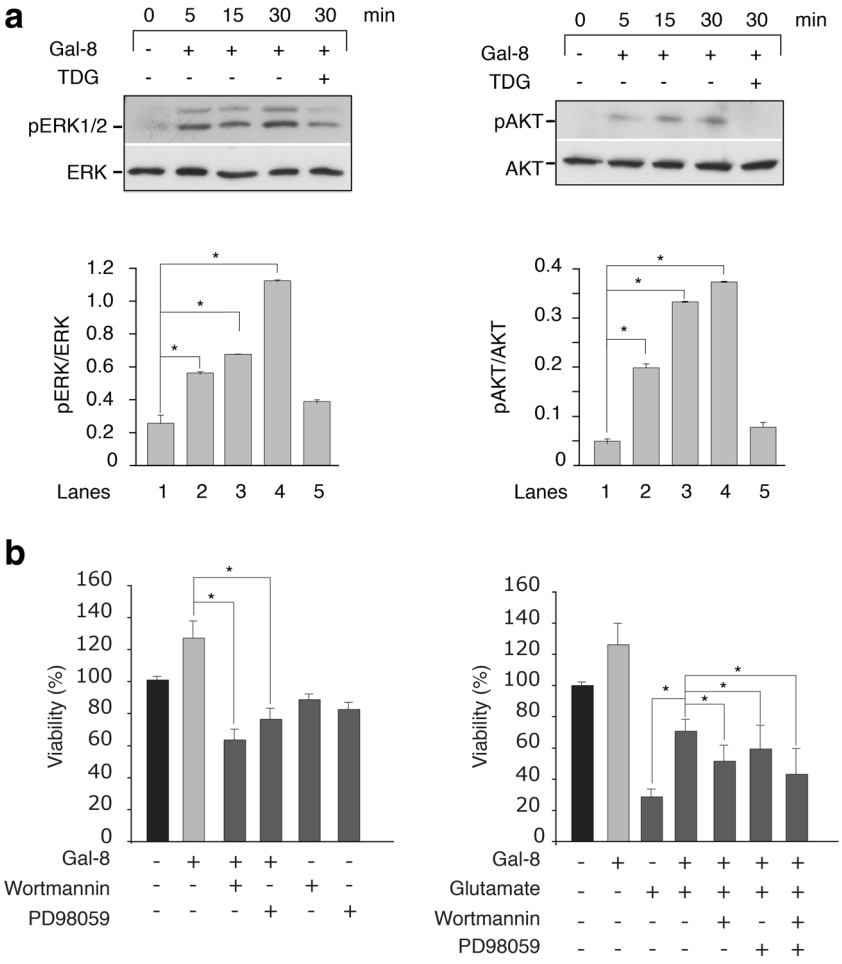

Fig. 5 MAPK ERK1/2 and PI3K/AKT signaling pathways mediate Gal-8 neuroprotection. **a** Gal-8 activates ERK1/2 and AKT in a TDG sensitive manner. Hippocampal neurons incubated with Gal-8 (1.5 μM) for the indicated periods of time showed increased levels of pERK1/2 and pAKT-active/phosphorylated forms, except upon TDG preincubation. **b** Inhibitors of ERK1/2 (PD98059; 25 μM) and PI3K/AKT (wortmannin; 50 nM) counteracted the neuroprotective effect of Gal-8 against glutamate (15 μM) treatment. (*$p < 0.01$ ANOVA; $n = 3$, each of the three experiments in triplicate)

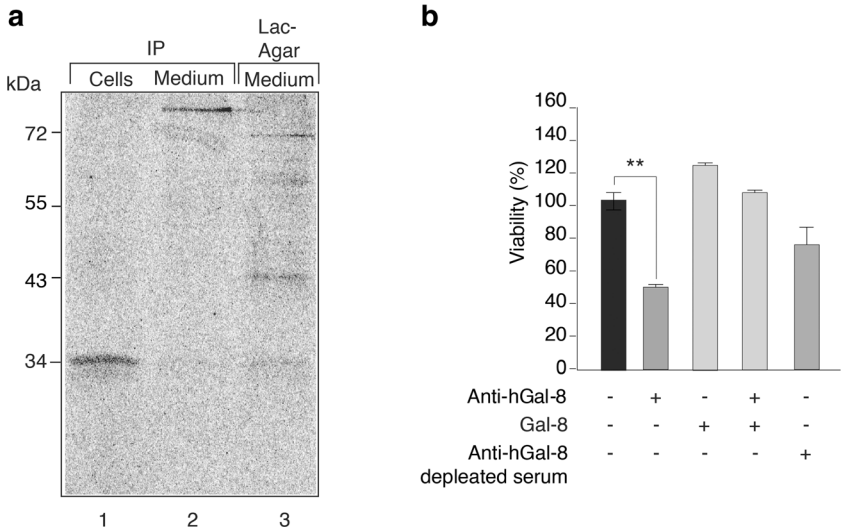

Fig. 6 Gal-8 endogenously expressed and secreted by hippocampal neurons is neuroprotective. **a** Immunoprecipitation of newly synthesized Gal-8 in primary hippocampal neurons and culture medium. Hippocampal neurons were metabolically labeled with 100 μCi ml^{-1} [^{35}S] methionine/cysteine during 4 h, and the expression of Gal-8 was analyzed by immunoprecipitation from cell extracts (lane 1) and media (lane 2). The 34 kDa band in the fluorography corresponds to the molecular mass of Gal-8. Precipitation from media with lactose-agarose beads (lane 3) also revealed a band of similar molecular mass. **b** Anti-Gal-8 function-blocking antibodies, affinity purified from serum of SLE patients, decreased survival of hippocampal neurons, whereas antibodies preincubated with an excess of Gal-8 (1.5 μM), as well as anti-Gal-8-depleted serum show no effect (**$p < 0.001$ ANOVA; $n = 3$, each of three experiments in triplicate)

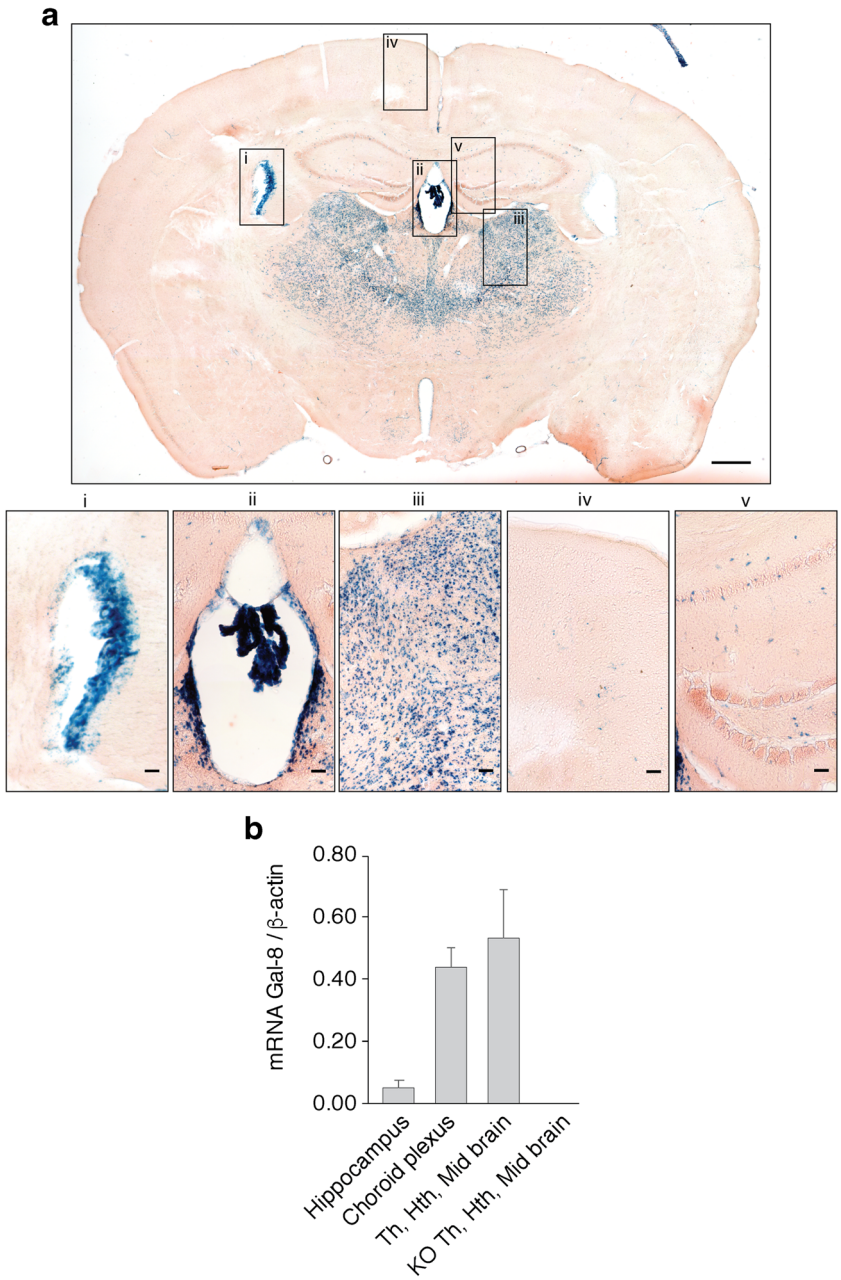

Fig. 7 Gal-8 expression in mouse brain. **a** β-gal staining of mouse brain indicates high expression levels of Gal-8 in the choroid plexus of the lateral (i) and dorsal 3rd ventricle (ii) and thalamus (iii), while hippocampus (iv) and cortex (v) show few scattered cells with β-gal staining reflecting Gal-8 expression (bars, 1 mm in the upper global figure and 100 μm in the lower panels of insets at higher magnifications). **b** Real-time PCR confirms that Gal-8 is expressed at low levels in hippocampus compared with both the choroid plexus and the combined regions of the thalamus (Th), hipothalamus (HTh), and midbrain (Mbrain). Control sample from the KO mouse shows no transcript detection

found in acute and chronic brain diseases. Gal-8-mediated neuroprotection involves activation of selective β1-integrins and MAPK-ERK1/2 and PI3K/AKT signaling pathways and can be blocked by human anti-Gal-8 autoantibodies.

We show that Gal-8 improves the survival of hippocampal neurons challenged with nutrient restriction, glutamate, hydrogen peroxide and Aβo. This neuroprotective effect involves Gal-8 interactions with cell surface glycoconjugates, as it can be effectively counteracted with TDG that blocks galectin-glycan interactions [28, 30]. Glutamate and hydrogen peroxide likely reproduce in vitro conditions affecting neurons during acute ischemia [55, 56]. Oxidative stress has also been involved in neurodegenerative diseases, including Alzheimer's disease, Parkinson, and amyothophic lateral sclerosis [10]. Aβ species induce neuronal death through glutamate excitotoxicity, activation of caspase-3 [60], oxidative stress [61], and alterations in calcium homeostasis [62] and Wnt signaling [57]. Our results not only show that Gal-8 can counteract all these harmful conditions but also that its protective action can be effective even 2 h after the exposure to the neurotoxic challenge. Therefore, Gal-8 might be suitable to ameliorate neuronal damage under acute damaging conditions, such as stroke, where a time-window is determinant to start treatment [63].

Integrins are well-known mediators of neuronal survival and protection against damaging conditions such as ischemia

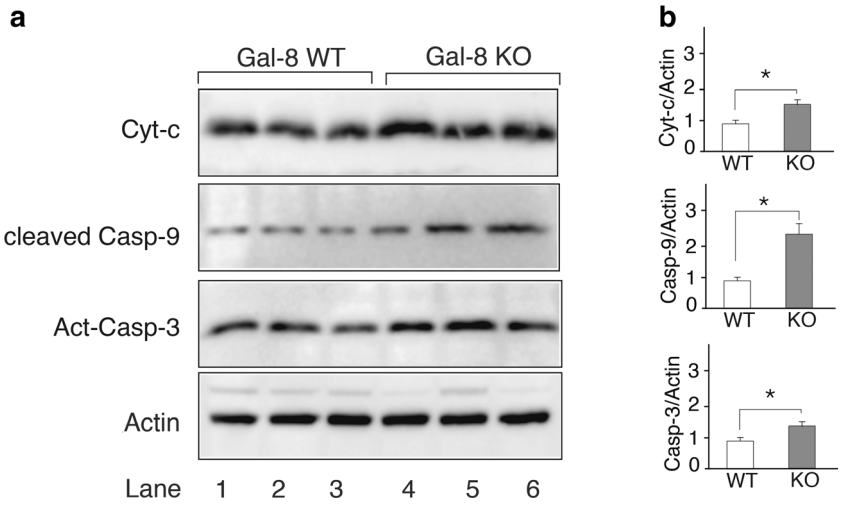

Fig. 8 Gal-8 protects hippocampus against damage from H_2O_2 in vivo. Western blots of caspase-9, caspase-3, and released cytochrome *c* in hippocampal homogenates from three WT and three Gal-8-KO mice analyzed 48 h after stereotaxic injection of H_2O_2 (50 μM) or PBS into the hippocampus. The graph represents the densitometric analysis of bands relative to a whole analysis of three WT and three Gal-8-KO mice. Bars represent the mean ± SEM. (*$p < 0.05$ student *t*; $n = 3$)

and excitotoxicity [31–33, 64]. Integrins also contribute to neuronal regeneration after physical injury or neuropathy [64]. Previous studies mainly focused on integrin activators found in ECM, such as fibronectin [35, 65–67] and laminin [31, 32, 68]. However, Gal-8 has been shown to be equipotent to fibronectin in selective β1-integrin activation [29], particularly engaging α5β1 [28, 30, 46]. Our pull-down experiments reveal that Gal-8 binds α3 and α5 but not α2 and α4 integrins in hippocampal neurons. Activation of α5β1 is known to improve neuronal survival [34]. Our results with an antibody that recognizes only the activated conformation of β1 integrins [34, 58] show that Gal-8 activates β1 integrins in hippocampal neurons. We also show that anti-β1 function-blocking antibodies decrease the neuroprotector effect of Gal-8. Even though the contribution of other cell surface interactions cannot be discarded, all these results crucially involve selected β1 integrins, including α5β1, in the neuroprotective effects of Gal-8.

Our results show that Gal-8 activates ERK1/2 and PI3K/AKT signaling in primary hippocampal neurons, with ERK1/2 activation seemingly downstream PI3K as judged by its abrogation with wortmannin. Cross activation and potentiation between ERK1/2 and PI3K/AKT signaling occur in both senses depending on the cellular context [69, 70]. We also show that Gal-8 neuroprotection can be counteracted with ERK1/2 and PI3K inhibitors. ERK1/2 and PI3K/AKT signaling pathways are well-documented survival pathways in neurons [2], protecting against apoptotic inducers such as lack of nutrients, glutamate, and Aβ peptide [71, 72]. Both ERK1/2 and PI3K/AKT survival pathways counteract pro-apoptotic proteins and increase the expression of apoptotic-preventive proteins [2]. For instance, ERK1/2 and AKT activities converge upon phosphorylation-mediated inhibition of GSK3β and pro-apoptotic BAD proteins, as well as on the activation of the transcription factor CREB that increases the anti-apoptotic expression of Bcl-2 and BDNF [73, 74]. AKT also activates NF-κB that modulates the transcription of Bcl-2 and Bcl-x [74]. Furthermore, caspase-9 is inhibited by PI3K phosphorylation [75, 76]. The window of Gal-8 effectiveness, expanding 2 h after incubation with the harmful conditions, implies that at some point the downstream elements of the damaging pathways become irreversibly committed towards the apoptotic process. Gal-8 might only prevent the initial steps by triggering the counteracting ERK1/2 and AKT protective signaling.

Gal-1, Gal-3, Gal-4, Gal-8, and Gal-9 have been reported as the main galectins expressed in the CNS [18, 77], where they can have complementary roles and also complementary therapeutic potentials. Gal-4 is expressed by hippocampal neurons in primary culture and is required for axonal sorting and clustered organization of NCAM L1 glycoprotein at the plasma membrane, implied in axonal growth [78]. Gal-1 and Gal-3 have long been studied in the central and peripheral nervous systems, but there is no much information regarding survival or protecting effects directly on neurons [79]. Gal-1 has been described to induce degeneration of neuronal processes associated with overexpression of the p75 neurotrophic receptor [80]. However, contradicting results reported no direct effect of Gal-1 upon neuron survival or neuronal processes in culture, whereas it prevents inflammation-induced neurodegeneration through selective deactivation of microglial cells [81]. In primary cultures of cerebral granular neurons, Gal-1 has been found to provide neuroprotection specifically against glutamate but not to hydrogen peroxide or to other toxic insults [82]. Oxidized or monomeric Gal-1, having little lectin activity, promotes peripheral axonal growth and regeneration by indirectly engaging macrophage function [83, 84]. In vivo, Gal-1 improves recovery after focal brain ischemia [85]. Gal-3 might also be a neuronal survival factor, as it promotes neural cell adhesion, neurite growth [86], and protection against Aβ-induced neuronal death [87]. Additional effects of Gal-1 and Gal-3 in the CNS include modulation of

neuronal stem cells, re-myelination and regeneration processes post-peripheral injury, as well as modulation of immune reactions against the CNS in autoimmune diseases [79]. Gal-3 also negatively regulates hippocampal-mediated memory [88]. As mentioned, Gal-8 has immunosuppresive actions that can protect CNS from autoimmune attacks [19]. Here, we add Gal-8 as an example of a galectin able to protect neurons against a variety of injuring conditions, offering a 2-h time-lapse period for an effective treatment intervention against acute damage.

Galectins are secreted by some yet unknown pathway likely including exosomal release and can extracellularly act as autocrine regulation factors [16, 89]. We presented evidence indicating that hippocampal neurons in primary culture not only respond to exogenously added Gal-8 but also secrete endogenous Gal-8 competent as neurosurvival factor. Our immunoprecipitation experiments revealed newly synthesized Gal-8 in the media of metabolically labeled hippocampal neurons. We also show that human function-blocking anti-Gal-8 antibodies isolated from a SLE patient decrease the survival of these neurons. Therefore, neurons themselves can generate a Gal-8-protecting environment, which becomes ineffective in the presence of human anti-Gal-8 autoantibodies such as those generated by patients with SLE [28, 30, 37] or MS [19].

We also analyzed the expression and neuroprotective potential of Gal-8 in adult mouse brain taken advantage of Gal-8 KO mice. A β-galactosidase reporter inserted downstream the Gal-8 promoter and qPCR analysis both show low levels of Gal-8 expression in the hippocampus compared with the higher levels displayed by the thalamus and choroid plexus. However, despite the low level of Gal-8 expression in hippocampus, we found a clear neuroprotection effect provided by Gal-8 in this region. Stereotaxic injection of H_2O_2 into the hippocampus induced higher levels of several apoptotic markers in Gal-8 KO compared with wild-type mice. This observation suggests that Gal-8 can exert neuroprotection in adult brain even in regions where its expression is relatively low. It is possible that neurons all over the brain constantly receive Gal-8 through the CSF. The choroid plexus that generates the CSF has high levels of Gal-8 expression, and we have previously detected Gal-8 in human CSF [19]. Components of CSF can penetrate into the brain parenchyma along arterial paravascular spaces [90–92]. Therefore, a main source of Gal-8 bathing the adult brain can be the CSF generated by the choroid plexus [19].

The role of Gal-8 in the brain has previously been related to modulation of the immune-surveillance system that patrols the CNS, thus protecting against autoimmune conditions [19]. We have described that Gal-8 KO mice develop exacerbated symptoms of EAE, correlated with enhanced inflammation and demyelination lesions in affected CNS areas, while Gal-8 treatment of wild-type mice ameliorates the disease symptoms [19]. Furthermore, patients with multiple sclerosis generate function-blocking anti-Gal-8 antibodies that predict clinical disability reflecting neuronal damage [19]. Anti-Gal-8 antibodies can be found not only in the circulation but also in the CSF of these patients [19]. These observations can be explained in part by a role of Gal-8 as immunosuppressive factor [19, 25, 93], which modulate the balance of Th17 and Th1 polarization and their respective Tregs [19]. Anti-Gal-8 autoantibodies have been associated with lymphopenia in SLE and can also been found in rheumatoid arthritis and other inflammatory disorders [37]. Antibodies can reach the brain parenchyma from the circulation under a variety of pathogenic conditions that breach the blood brain barrier, including severe infections, or can be directly produced in the brain when immune cells penetrate into the organ during autoimmune conditions [94]. Our present results add a new function of Gal-8 in the brain, which is to contribute to generate a protective environment by promoting β1-integrin-dependent survival systems in neurons. Function-blocking anti-Gal-8 antibodies generated under autoimmune or inflammatory conditions might eventually reduce such Gal-8-mediated neuroprotection.

Funding This study is financially supported by the Programa de Apoyo a Centros con Financiamiento Basal AFB 170005 (A.G, N.C.I, A.S., C.M., F.B, S.E, E.P., J.G.) and AFB 170004 (C.T.R) from the Comisión Nacional de Investigación Científica y Tecnológica (CONICYT), Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) PhD fellowship No. 3120061 (E.P.), FONDECYT No. 1131122 (A.S.), CONICYT PhD Fellowship (F.B, S.E.), FWIS UNESCO-L'Oreal fellowship (F.B.), FONDECYT 11170546 and CONICYT PAI 77170091 (C.T.R.), and FONDECYT 1160513 (L.M.).

References

1. Benn SC, Woolf CJ (2004) Adult neuron survival strategies—slamming on the brakes. Nat Rev Neurosci 5(9):686–700. https://doi.org/10.1038/nrn1477
2. Kole AJ, Annis RP, Deshmukh M (2013) Mature neurons: equipped for survival. Cell Death Dis 4:e689. https://doi.org/10.1038/cddis.2013.220
3. Fricker M, Tolkovsky AM, Borutaite V, Coleman M, Brown GC (2018) Neuronal cell death. Physiol Rev 98(2):813–880. https://doi.org/10.1152/physrev.00011.2017
4. Schwartz N, Stock AD, Putterman C (2019) Neuropsychiatric lupus: new mechanistic insights and future treatment directions. Nat Rev Rheumatol 15:137–152. https://doi.org/10.1038/s41584-018-0156-8
5. Gonzalez A, Massardo L (2018) Antibodies and the brain: antiribosomal P protein antibody and the clinical effects in patients with systemic lupus erythematosus. Curr Opin Neurol 31(3):300–305. https://doi.org/10.1097/WCO.0000000000000549
6. Mackay M, Vo A, Tang CC, Small M, Anderson EW, Ploran EJ, Storbeck J, Bascetta B et al (2019) Metabolic and microstructural alterations in the SLE brain correlate with cognitive impairment. J Clin Invest Insight 4(1). https://doi.org/10.1172/jci.insight.124002
7. Mader S, Brimberg L, Diamond B (2017) The role of brain-reactive autoantibodies in brain pathology and cognitive impairment. Front Immunol 8:1101. https://doi.org/10.3389/fimmu.2017.01101

8. Benussi L, Binetti G, Ghidoni R (2017) Loss of neuroprotective factors in neurodegenerative dementias: the end or the starting point? Front Neurosci 11:672. https://doi.org/10.3389/fnins.2017.00672
9. Tapia-Rojas C, Inestrosa NC (2018) Wnt signaling loss accelerates the appearance of neuropathological hallmarks of Alzheimer's disease in J20-APP transgenic and wild-type mice. J Neurochem 144(4):443–465. https://doi.org/10.1111/jnc.14278
10. Andersen JK (2004) Oxidative stress in neurodegeneration: cause or consequence? Nat Med 10(Suppl):S18–S25. https://doi.org/10.1038/nrn1434
11. Vasta GR, Feng C, Gonzalez-Montalban N, Mancini J, Yang L, Abernathy K, Frost G, Palm C (2017) Functions of galectins as 'self/non-self'-recognition and effector factors. Pathog Dis 75 (5). doi:https://doi.org/10.1093/femspd/ftx046
12. Rabinovich GA, Croci DO (2012) Regulatory circuits mediated by lectin-glycan interactions in autoimmunity and cancer. Immunity 36(3):322–335. https://doi.org/10.1016/j.immuni.2012.03.004
13. Kaltner H, Gabius HJ (2012) A toolbox of lectins for translating the sugar code: the galectin network in phylogenesis and tumors. Histol Histopathol 27(4):397–416
14. Siew JJ, Chern Y (2018) Microglial lectins in health and neurological diseases. Front Mol Neurosci 11:158. https://doi.org/10.3389/fnmol.2018.00158
15. Thurston TL, Wandel MP, von Muhlinen N, Foeglein A, Randow F (2012) Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. Nature 482(7385):414–418. https://doi.org/10.1038/nature10744
16. Popa SJ, Stewart SE, Moreau K (2018) Unconventional secretion of annexins and galectins. Semin Cell Dev Biol 83:42–50. https://doi.org/10.1016/j.semcdb.2018.02.022
17. Nabi IR, Shankar J, Dennis JW (2015) The galectin lattice at a glance. J Cell Sci 128(13):2213–2219. https://doi.org/10.1242/jcs.151159
18. Stancic M, van Horssen J, Thijssen VL, Gabius HJ, van der Valk P, Hoekstra D, Baron W (2011) Increased expression of distinct galectins in multiple sclerosis lesions. Neuropathol Appl Neurobiol 37(6):654–671. https://doi.org/10.1111/j.1365-2990.2011.01184.x
19. Pardo E, Carcamo C, Uribe-San Martin R, Ciampi E, Segovia-Miranda F, Curkovic-Pena C, Montecino F, Holmes C et al (2017) Galectin-8 as an immunosuppressor in experimental autoimmune encephalomyelitis and a target of human early prognostic antibodies in multiple sclerosis. PLoS One 12(6):e0177472. https://doi.org/10.1371/journal.pone.0177472
20. Elola MT, Ferragut F, Cardenas Delgado VM, Nugnes LG, Gentilini L, Laderach D, Troncoso MF, Compagno D et al (2014) Expression, localization and function of galectin-8, a tandem-repeat lectin, in human tumors. Histol Histopathol 29(9):1093–1105
21. Jia J, Abudu YP, Claude-Taupin A, Gu Y, Kumar S, Choi SW, Peters R, Mudd MH et al (2018) Galectins control mTOR in response to endomembrane damage. Mol Cell 70(1):120–135e128. https://doi.org/10.1016/j.molcel.2018.03.009
22. Staring J, von Castelmur E, Blomen VA, van den Hengel LG, Brockmann M, Baggen J, Thibaut HJ, Nieuwenhuis J et al (2017) PLA2G16 represents a switch between entry and clearance of Picornaviridae. Nature 541(7637):412–416. https://doi.org/10.1038/nature21032
23. Falcon B, Noad J, McMahon H, Randow F, Goedert M (2018) Galectin-8-mediated selective autophagy protects against seeded tau aggregation. J Biol Chem 293(7):2438–2451. https://doi.org/10.1074/jbc.M117.809293
24. Vicuna L, Pardo E, Curkovic C, Doger R, Oyanadel C, Metz C, Massardo L, Gonzalez A et al (2013) Galectin-8 binds to LFA-1, blocks its interaction with ICAM-1 and is counteracted by anti-Gal-8 autoantibodies isolated from lupus patients. Biol Res 46(3):275–280. https://doi.org/10.4067/S0716-97602013000300008
25. Sampson JF, Suryawanshi A, Chen WS, Rabinovich GA, Panjwani N (2016) Galectin-8 promotes regulatory T-cell differentiation by modulating IL-2 and TGFbeta signaling. Immunol Cell Biol 94(2):213–219. https://doi.org/10.1038/icb.2015.72
26. Hadari YR, Arbel-Goren R, Levy Y, Amsterdam A, Alon R, Zakut R, Zick Y (2000) Galectin-8 binding to integrins inhibits cell adhesion and induces apoptosis. J Cell Sci 113(Pt 13):2385–2397
27. Nishi N, Shoji H, Seki M, Itoh A, Miyanaka H, Yuube K, Hirashima M, Nakamura T (2003) Galectin-8 modulates neutrophil function via interaction with integrin alphaM. Glycobiology 13(11):755–763
28. Carcamo C, Pardo E, Oyanadel C, Bravo-Zehnder M, Bull P, Caceres M, Martinez J, Massardo L et al (2006) Galectin-8 binds specific beta1 integrins and induces polarized spreading highlighted by asymmetric lamellipodia in Jurkat T cells. Exp Cell Res 312(4):374–386. https://doi.org/10.1016/j.yexcr.2005.10.025
29. Levy Y, Arbel-Goren R, Hadari YR, Eshhar S, Ronen D, Elhanany E, Geiger B, Zick Y (2001) Galectin-8 functions as a matricellular modulator of cell adhesion. J Biol Chem 276(33):31285–31295
30. Norambuena A, Metz C, Vicuna L, Silva A, Pardo E, Oyanadel C, Massardo L, Gonzalez A et al (2009) Galectin-8 induces apoptosis in Jurkat T cells by phosphatidic acid-mediated ERK1/2 activation supported by protein kinase A down-regulation. J Biol Chem 284(19):12670–12679. https://doi.org/10.1074/jbc.M808949200
31. Gary DS, Mattson MP (2001) Integrin signaling via the PI3-kinase-Akt pathway increases neuronal resistance to glutamate-induced apoptosis. J Neurochem 76(5):1485–1496
32. Gary DS, Milhavet O, Camandola S, Mattson MP (2003) Essential role for integrin linked kinase in Akt-mediated integrin survival signaling in hippocampal neurons. J Neurochem 84(4):878–890
33. Murase S, Owens DF, McKay RD (2011) In the newborn hippocampus, neurotrophin-dependent survival requires spontaneous activity and integrin signaling. J Neurosci 31(21):7791–7800. https://doi.org/10.1523/JNEUROSCI.0202-11.2011
34. Gibson RM, Craig SE, Heenan L, Tournier C, Humphries MJ (2005) Activation of integrin alpha5beta1 delays apoptosis of Ntera2 neuronal cells. Mol Cell Neurosci 28(3):588–598. https://doi.org/10.1016/j.mcn.2004.11.004
35. Wang J, Yin L, Chen Z (2013) Neuroprotective role of fibronectin in neuron-glial extrasynaptic transmission. Neural Regen Res 8(4):376–382. https://doi.org/10.3969/j.issn.1673-5374.2013.04.010
36. Sakai T, Johnson KJ, Murozono M, Sakai K, Magnuson MA, Wieloch T, Cronberg T, Isshiki A et al (2001) Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin-wound healing and hemostasis. Nat Med 7(3):324–330. https://doi.org/10.1038/85471
37. Massardo L, Metz C, Pardo E, Mezzano V, Babul M, Jarpa E, Guzman AM, Andre S et al (2009) Autoantibodies against galectin-8: their specificity, association with lymphopenia in systemic lupus erythematosus and detection in rheumatoid arthritis and acute inflammation. Lupus 18(6):539–546. https://doi.org/10.1177/0961203308099973
38. Pardo E, Carcamo C, Massardo L, Mezzano V, Jacobelli S, Gonzalez A, Soza A (2006) Antibodies against galectin-8 in patients with systemic lupus erythematosus. Rev Med Chil 134(2):159–166
39. Caceres A, Binder LI, Payne MR, Bender P, Rebhun L, Steward O (1984) Differential subcellular localization of tubulin and the microtubule-associated protein MAP2 in brain tissue as revealed by immunocytochemistry with monoclonal hybridoma antibodies. J Neurosci 4(2):394–410
40. Ramirez VT, Ramos-Fernandez E, Henriquez JP, Lorenzo A, Inestrosa NC (2016) Wnt-5a/Frizzled9 receptor signaling through

the Galphao/Gbetagamma complex regulates dendritic spine formation. J Biol Chem 291:19092–19107. https://doi.org/10.1074/jbc.M116.722132
41. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65(1–2):55–63
42. Farias GG, Valles AS, Colombres M, Godoy JA, Toledo EM, Lukas RJ, Barrantes FJ, Inestrosa NC (2007) Wnt-7a induces presynaptic colocalization of alpha 7-nicotinic acetylcholine receptors and adenomatous polyposis coli in hippocampal neurons. J Neurosci 27(20):5313–5325. https://doi.org/10.1523/JNEUROSCI.3934-06.2007
43. Dinamarca MC, Weinstein D, Monasterio O, Inestrosa NC (2011) The synaptic protein neuroligin-1 interacts with the amyloid beta-peptide. Is there a role in Alzheimer's disease? Biochemistry 50(38):8127–8137. https://doi.org/10.1021/bi201246t
44. Silva-Alvarez C, Arrazola MS, Godoy JA, Ordenes D, Inestrosa NC (2013) Canonical Wnt signaling protects hippocampal neurons from Abeta oligomers: role of non-canonical Wnt-5a/Ca(2+) in mitochondrial dynamics. Front Cell Neurosci 7:97. https://doi.org/10.3389/fncel.2013.00097
45. Bravo-Zehnder M, Toledo EM, Segovia-Miranda F, Serrano FG, Benito MJ, Metz C, Retamal C, Alvarez A et al (2015) Anti-ribosomal P protein autoantibodies from patients with neuropsychiatric lupus impair memory in mice. Arthritis Rheum 67(1):204–214. https://doi.org/10.1002/art.38900
46. Oyanadel C, Holmes C, Pardo E, Retamal C, Shaughnessy R, Smith P, Cortes P, Bravo-Zehnder M et al (2018) Galectin-8 induces partial epithelial-mesenchymal transition with invasive tumorigenic capabilities involving a FAK/EGFR/proteasome pathway in Madin-Darby canine kidney cells. Mol Biol Cell 29(5):557–574. https://doi.org/10.1091/mbc.E16-05-0301
47. Valenzuela DM, Murphy AJ, Frendewey D, Gale NW, Economides AN, Auerbach W, Poueymirou WT, Adams NC et al (2003) High-throughput engineering of the mouse genome coupled with high-resolution expression analysis. Nat Biotechnol 21(6):652–659. https://doi.org/10.1038/nbt822
48. Poueymirou WT, Auerbach W, Frendewey D, Hickey JF, Escaravage JM, Esau L, Dore AT, Stevens S et al (2007) F0 generation mice fully derived from gene-targeted embryonic stem cells allowing immediate phenotypic analyses. Nat Biotechnol 25(1):91–99. https://doi.org/10.1038/nbt1263
49. Segovia-Miranda F, Serrano F, Dyrda A, Ampuero E, Retamal C, Bravo-Zehnder M, Parodi J, Zamorano P et al (2015) Pathogenicity of lupus anti-ribosomal p antibodies: role of cross-reacting neuronal surface p-antigen in glutamatergic transmission and plasticity. Arthritis Rheum 67:1598–1610. https://doi.org/10.1002/art.39081
50. Vargas JY, Fuenzalida M, Inestrosa NC (2014) In vivo activation of Wnt signaling pathway enhances cognitive function of adult mice and reverses cognitive deficits in an Alzheimer's disease model. J Neurosci 34(6):2191–2202. https://doi.org/10.1523/JNEUROSCI.0862-13.2014
51. Feeney CJ, Frantseva MV, Carlen PL, Pennefather PS, Shulyakova N, Shniffer C, Mills LR (2008) Vulnerability of glial cells to hydrogen peroxide in cultured hippocampal slices. Brain Res 1198:1–15. https://doi.org/10.1016/j.brainres.2007.12.049
52. Gerich FJ, Funke F, Hildebrandt B, Fasshauer M, Muller M (2009) H(2)O(2)-mediated modulation of cytosolic signaling and organelle function in rat hippocampus. Pflugers Arch 458(5):937–952. https://doi.org/10.1007/s00424-009-0672-0
53. Tapia-Rojas C, Carvajal FJ, Mira RG, Arce C, Lerma-Cabrera JM, Orellana JA, Cerpa W, Quintanilla RA (2018) Adolescent binge alcohol exposure affects the brain function through mitochondrial impairment. Mol Neurobiol 55(5):4473–4491. https://doi.org/10.1007/s12035-017-0613-4
54. Patel NJ, Chen MJ, Russo-Neustadt AA (2010) Norepinephrine and nitric oxide promote cell survival signaling in hippocampal neurons. Eur J Pharmacol 633(1–3):1–9. https://doi.org/10.1016/j.ejphar.2010.01.012
55. Chamorro A, Dirnagl U, Urra X, Planas AM (2016) Neuroprotection in acute stroke: Targeting excitotoxicity, oxidative and nitrosative stress, and inflammation. Lancet Neurol 15(8):869–881. https://doi.org/10.1016/S1474-4422(16)00114-9
56. Lai TW, Zhang S, Wang YT (2014) Excitotoxicity and stroke: identifying novel targets for neuroprotection. Prog Neurobiol 115:157–188. https://doi.org/10.1016/j.pneurobio.2013.11.006
57. De Ferrari GV, Chacon MA, Barria MI, Garrido JL, Godoy JA, Olivares G, Reyes AE, Alvarez A et al (2003) Activation of Wnt signaling rescues neurodegeneration and behavioral impairments induced by beta-amyloid fibrils. Mol Psychiatry 8(2):195–208. https://doi.org/10.1038/sj.mp.4001208
58. Whittard JD, Akiyama SK (2001) Activation of beta1 integrins induces cell-cell adhesion. Exp Cell Res 263(1):65–76. https://doi.org/10.1006/excr.2000.5099
59. Massardo L, Suarez-Almazor ME, Cardiel MH, Nava A, Levy RA, Laurindo I, Soriano ER, Acevedo-Vazquez E et al (2009) Management of patients with rheumatoid arthritis in Latin America: a consensus position paper from Pan-American League of Associations of Rheumatology and Grupo Latino Americano De Estudio De Artritis Reumatoide. J Clin Rheumatol 15(4):203–210. https://doi.org/10.1097/RHU.0b013e3181a90cd8
60. Takuma H, Tomiyama T, Kuida K, Mori H (2004) Amyloid beta peptide-induced cerebral neuronal loss is mediated by caspase-3 in vivo. J Neuropathol Exp Neurol 63(3):255–261
61. Behl C, Davis JB, Lesley R, Schubert D (1994) Hydrogen peroxide mediates amyloid beta protein toxicity. Cell 77(6):817–827
62. Resende R, Pereira C, Agostinho P, Vieira AP, Malva JO, Oliveira CR (2007) Susceptibility of hippocampal neurons to Abeta peptide toxicity is associated with perturbation of Ca2+ homeostasis. Brain Res 1143:11–21. https://doi.org/10.1016/j.brainres.2007.01.071
63. Hacke W, Kaste M, Bluhmki E, Brozman M, Davalos A, Guidetti D, Larrue V, Lees KR et al (2008) Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. N Engl J Med 359(13):1317–1329. https://doi.org/10.1056/NEJMoa0804656
64. Gardiner NJ (2011) Integrins and the extracellular matrix: key mediators of development and regeneration of the sensory nervous system. Dev Neurobiol 71(11):1054–1072. https://doi.org/10.1002/dneu.20950
65. Zhao LR, Spellman S, Kim J, Duan WM, McCarthy JB, Low WC (2005) Synthetic fibronectin peptide exerts neuroprotective effects on transient focal brain ischemia in rats. Brain Res 1054(1):1–8. https://doi.org/10.1016/j.brainres.2005.04.056
66. Farias E, Lu M, Li X, Schnapp LM (2005) Integrin alpha8beta1-fibronectin interactions promote cell survival via PI3 kinase pathway. Biochem Biophys Res Commun 329(1):305–311. https://doi.org/10.1016/j.bbrc.2005.01.125
67. Tate CC, Garcia AJ, LaPlaca MC (2007) Plasma fibronectin is neuroprotective following traumatic brain injury. Exp Neurol 207(1):13–22. https://doi.org/10.1016/j.expneurol.2007.05.008
68. Chen ZL, Indyk JA, Strickland S (2003) The hippocampal laminin matrix is dynamic and critical for neuronal survival. Mol Biol Cell 14(7):2665–2676. https://doi.org/10.1091/mbc.E02-12-0832
69. Duckworth BC, Cantley LC (1997) Conditional inhibition of the mitogen-activated protein kinase cascade by wortmannin. Dependence on signal strength. J Biol Chem 272(44):27665–27670
70. Wang CC, Cirit M, Haugh JM (2009) PI3K-dependent cross-talk interactions converge with Ras as quantifiable inputs integrated by Erk. Mol Syst Biol 5:246. https://doi.org/10.1038/msb.2009.4
71. Morrison RS, Kinoshita Y, Johnson MD, Ghatan S, Ho JT, Garden G (2002) Neuronal survival and cell death signaling pathways. Adv Exp Med Biol 513:41–86

72. Rossler OG, Giehl KM, Thiel G (2004) Neuroprotection of immortalized hippocampal neurones by brain-derived neurotrophic factor and Raf-1 protein kinase: role of extracellular signal-regulated protein kinase and phosphatidylinositol 3-kinase. J Neurochem 88(5): 1240–1252
73. Parcellier A, Tintignac LA, Zhuravleva E, Hemmings BA (2008) PKB and the mitochondria: AKTing on apoptosis. Cell Signal 20(1):21–30. https://doi.org/10.1016/j.cellsig.2007.07.010
74. Hetman M, Gozdz A (2004) Role of extracellular signal regulated kinases 1 and 2 in neuronal survival. Eur J Biochem 271(11):2050–2055. https://doi.org/10.1111/j.1432-1033.2004.04133.x
75. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC (1998) Regulation of cell death protease caspase-9 by phosphorylation. Science 282(5392):1318–1321
76. Duronio V (2008) The life of a cell: apoptosis regulation by the PI3K/PKB pathway. Biochem J 415(3):333–344. https://doi.org/10.1042/BJ20081056
77. John S, Mishra R (2016) mRNA transcriptomics of galectins unveils heterogeneous organization in mouse and human brain. Front Mol Neurosci 9:139. https://doi.org/10.3389/fnmol.2016.00139
78. Velasco S, Diez-Revuelta N, Hernandez-Iglesias T, Kaltner H, Andre S, Gabius HJ, Abad-Rodriguez J (2013) Neuronal Galectin-4 is required for axon growth and for the organization of axonal membrane L1 delivery and clustering. J Neurochem 125(1): 49–62. https://doi.org/10.1111/jnc.12148
79. Chen HL, Liao F, Lin TN, Liu FT (2014) Galectins and neuroinflammation. Adv Neurobiol 9:517–542. https://doi.org/10.1007/978-1-4939-1154-7_24
80. Plachta N, Annaheim C, Bissiere S, Lin S, Ruegg M, Hoving S, Muller D, Poirier F et al (2007) Identification of a lectin causing the degeneration of neuronal processes using engineered embryonic stem cells. Nat Neurosci 10(6):712–719. https://doi.org/10.1038/nn1897
81. Starossom SC, Mascanfroni ID, Imitola J, Cao L, Raddassi K, Hernandez SF, Bassil R, Croci DO et al (2012) Galectin-1 deactivates classically activated microglia and protects from inflammation-induced neurodegeneration. Immunity 37(2):249–263. https://doi.org/10.1016/j.immuni.2012.05.023
82. Lekishvili T, Hesketh S, Brazier MW, Brown DR (2006) Mouse galectin-1 inhibits the toxicity of glutamate by modifying NR1 NMDA receptor expression. Eur J Neurosci 24(11):3017–3025. https://doi.org/10.1111/j.1460-9568.2006.05207.x
83. Horie H, Kadoya T, Hikawa N, Sango K, Inoue H, Takeshita K, Asawa R, Hiroi T et al (2004) Oxidized galectin-1 stimulates macrophages to promote axonal regeneration in peripheral nerves after axotomy. J Neurosci 24(8):1873–1880. https://doi.org/10.1523/jneurosci.4483-03.2004
84. Horie H, Inagaki Y, Sohma Y, Nozawa R, Okawa K, Hasegawa M, Muramatsu N, Kawano H et al (1999) Galectin-1 regulates initial axonal growth in peripheral nerves after axotomy. J Neurosci 19(22):9964–9974
85. Ishibashi S, Kuroiwa T, Sakaguchi M, Sun L, Kadoya T, Okano H, Mizusawa H (2007) Galectin-1 regulates neurogenesis in the subventricular zone and promotes functional recovery after stroke. Exp Neurol 207(2):302–313. https://doi.org/10.1016/j.expneurol.2007.06.024
86. Pesheva P, Kuklinski S, Schmitz B, Probstmeier R (1998) Galectin-3 promotes neural cell adhesion and neurite growth. J Neurosci Res 54(5):639–654. https://doi.org/10.1002/(SICI)1097-4547(19981201)54:5<639::AID-JNR9>3.0.CO;2-2
87. Kim JY, Kim DH, Kim DS, Kim JH, Jeong SY, Jeon HB, Lee EH, Yang YS et al (2010) Galectin-3 secreted by human umbilical cord blood-derived mesenchymal stem cells reduces amyloid-beta42 neurotoxicity in vitro. FEBS Lett 584(16):3601–3608. https://doi.org/10.1016/j.febslet.2010.07.028
88. Chen YC, Ma YL, Lin CH, Cheng SJ, Hsu WL, Lee EH (2017) Galectin-3 negatively regulates hippocampus-dependent memory formation through inhibition of integrin signaling and Galectin-3 phosphorylation. Front Mol Neurosci 10:217. https://doi.org/10.3389/fnmol.2017.00217
89. Elola MT, Wolfenstein-Todel C, Troncoso MF, Vasta GR, Rabinovich GA (2007) Galectins: matricellular glycan-binding proteins linking cell adhesion, migration, and survival. Cell Mol Life Sci 64(13):1679–1700. https://doi.org/10.1007/s00018-007-7044-8
90. Brinker T, Stopa E, Morrison J, Klinge P (2014) A new look at cerebrospinal fluid circulation. FBCNS 11:10. https://doi.org/10.1186/2045-8118-11-10
91. Simon MJ, Iliff JJ (2016) Regulation of cerebrospinal fluid (CSF) flow in neurodegenerative, neurovascular and neuroinflammatory disease. Biochim Biophys Acta 1862(3):442–451. https://doi.org/10.1016/j.bbadis.2015.10.014
92. Iliff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, Benveniste H, Vates GE et al (2012) A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta. Sci Transl Med 4(147): 147ra111. https://doi.org/10.1126/scitranslmed.3003748
93. Sampson JF, Hasegawa E, Mulki L, Suryawanshi A, Jiang S, Chen WS, Rabinovich GA, Connor KM et al (2015) Galectin-8 ameliorates murine autoimmune ocular pathology and promotes a regulatory T cell response. PLoS One 10(6):e0130772. https://doi.org/10.1371/journal.pone.0130772
94. Stock AD, Gelb S, Pasternak O, Ben-Zvi A, Putterman C (2017) The blood brain barrier and neuropsychiatric lupus: new perspectives in light of advances in understanding the neuroimmune interface. Autoimmun Rev 16(6):612–619. https://doi.org/10.1016/j.autrev.2017.04.008