GM1 promotes TrkA-mediated neuroblastoma cell differentiation by occupying a plasma membrane domain different from TrkA

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† Dedicated to the memory of Dr. Riccardo Casellato, whose scientific contribution was fundamental to improve the synthesis of photoactivable gangliosides.

Running title:
TrkA and GM1 are in separate lipid rafts

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Abbreviations

Ganglioside nomenclature is in accordance with IUPAC-IUB recommendations (IUPAC-IUMBMB 1998).

BSA, bovine serum albumin

CT-B, cholera toxin-subunit B;

DRM, Detergent-Resistant Membrane

GM1, II^3Neu5Ac-Gg_4Cer, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-Cer;

GM1 oligosaccharide, II^3Neu5Ac-Gg_4, OligoGM1, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-Glc DMEM, Dulbecco's modified Eagles' medium;

FBS, fetal bovine serum;

HD, high density;

HPR, horseradish peroxidase;

HPTLC, high-performance silica gel thin-layer chromatography;

MAPK, mitogen-activated protein kinase;

N2a, Neuro2a cells;

NGF, nerve growth factor;

PBS, phosphate-buffered saline;

PNS, postnuclear supernatant;

PVDF, polyvinylidene difluoride;

RRID, Research Resource Identifier;

Trk, neurotrophin tyrosine kinase receptor;

Tyr490, tyrosine 490.

Abstract

Recently, we highlighted that the ganglioside GM1 promotes neuroblastoma cells differentiation by activating the TrkA receptor through the formation of a TrkA-GM1 oligosaccharide complex at the cell surface.

To study the TrkA-GM1 interaction, we synthesized two radioactive GM1 derivatives presenting a photoactivable nitrophenylazide group at the end of lipid moiety, **1** or at position 6 of external galactose, **2**; and a radioactive oligosaccharide portion of GM1 carrying the nitrophenylazide group at position 1 of glucose, **3**. The three compounds were singly administered to cultured neuroblastoma Neuro2a cells under established conditions that allows cell surface interactions. After UV activation of photoactivable compounds, the proteins were analyzed by PAGE separation. The formation of cross-linked TrkA-GM1 derivatives complexes were identified by both radioimaging and immunoblotting. Results indicated that the administration of compounds **2** and **3**, carrying the photoactivable group on the oligosaccharide, led to the formation of a radioactive TrkA complex, while the administration of compound **1** did not. This underlines that the TrkA-GM1 interaction directly involves the GM1 oligosaccharide, but not the ceramide.

To better understand how GM1 relates to the TrkA, we isolated plasma membrane lipid rafts. As expected, GM1 was found in the rigid detergent resistant fractions, while TrkA was found as a detergent soluble fraction component.

These results suggest that TrkA and GM1 belong to separate membrane domains: probably TrkA interacts by ‘flopping’ down its extracellular portion onto the membrane, approaching its interplay site to the oligosaccharide portion of GM1.

Introduction

Gangliosides, together with cholesterol, have been described as the driving molecules necessary to organize the cellular membranes with heterogeneous lipid distribution and to be responsible for the first steps of lipid rafts formation (Sonnino *et al*. 2006). In the lipid rafts, gangliosides can interact with proteins modulating their physical and functional properties (Sonnino *et al.* 2018).

The ganglioside GM1, marker of plasma membrane lipid rafts, has been described to play key functional roles in numerous events (Schengrund 2015; Ledeen and Wu 2015; Aureli *et al.* 2016). GM1 is one of the main component of the brain ganglioside pattern and many papers have elucidated its role as neurotrophic and neuroprotective molecule in *in vitro* and in *in vivo* models, comprising humans (Schneider *et al* 2010, 2013; Ledeen and Wu 2018a, 2018b).

GM1 exerts its well-known properties through the activation of plasma membrane receptors (Ledeend and Wu 2018a, 2018b), nevertheless, understanding whether the activity of a protein is determined by the ganglioside influence on membrane organization or by a direct ganglioside-protein interaction (Coskun and Simons 2011), is a hard challenge. However, the synthesis of tritium labeled and photoactivable gangliosides and their administration to cells in culture allows to study the ganglioside-protein interactions (Bisson and Montecucco 1981; Sonnino *et al.* 1989; Mauri *et al.* 2004). Indeed, after administration to the cells under dark conditions, these ganglioside derivatives are taken up by the cells becoming component of the plasma membranes and metabolically indistinguishable, from the endogenous compounds (Sonnino *et al.* 1989). Cell illumination produces a very reactive derivative containing nitrene capable to react with the molecules in the neighboring environment and forming a covalent linkage. Thus, proteins, interacting with the activated ganglioside, form a stable tritium labeled complex that can be identified by radioimaging technic after protein separation (Loberto *et al*. 2003; Mauri *et al.* 2004, Prioni *et al.* 2004; Chiricozzi *et al.* 2015). A detailed structural analysis of the complex allows to identify the involved protein.

Using this strategy in combination with other biochemical approaches, here, we studied how the ganglioside GM1 and the TrkA receptor interact in cultured neuroblastoma cells *in vitro*.

This interaction acts as a trigger for the activation of intracellular signaling cascades that ultimately produce cell differentiation and cell protection from toxic compounds. In this view neurotrophic and neuroprotective properties, long since attributed to GM1 (Mutoh *et al.* 1995; Chiricozzi *et al.* 2017), find, at least in part, a molecular explanation. In this paper, we present

data suggesting that GM1-TrkA interaction occurs between the extracellular moiety of TrkA that, probably lying upon the cells, reaches the oligosaccharide head of the GM1 whose ceramide portion is inserted in a membrane domain different from the receptor’s.

Methods

Materials

Commercial chemicals were of the highest purity available, common solvents were distilled before use and water was doubly distilled in a glass apparatus.

Cell culture plates and Transfectagro™ reduced serum medium were from Corning (Corning, NY, USA). Mouse neuroblastoma Neuro2a (N2a) cells (RRID: CVCL_0470), phosphate-buffered saline (PBS), HPR-conjugated cholera toxin subunit B (CT-B), o-phenylenediamine tablets, H_2O_2, vibrio cholerae sialidase, 2-propanol, Formic acid, Ponceau S, 3-(4,5,-dimethylthiazole-2yl)-2,5-diphenyltetrazolium bromide (MTT) and, bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's high glucose medium (DMEM-HG), Fetal Bovine Serum (FBS), L-Glutamine (L-Glut), Penicillin (10.000 Units/ml), Streptomycin and 30% acrylamide were from EuroClone (Paignton, UK). Rabbit anti-TrkA (RRID: AB_10695253), rabbit anti-phospho-TrkA (Tyr490) (RRID: AB_10235585), rabbit anti-p44/42 MAPK (ERK1/2) (RRID: AB_390779), rabbit anti-phospho-p44/42 MAPK (p-ERK1/2) (Thr202/Tyr204) (RRID: AB_2315112) and anti-rabbit IgG (RRID: AB_2099233) antibodies were from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-flotillin (RRID: AB_941621) was from Abcam (Cambridge, UK). Mouse anti-calnexin (RRID: AB_397884), goat anti-mouse IgG (H+L) antibody (RRID: AB_228307) and Hoechst-33342 fluorescent stain were from Thermo Fischer Scientific (Waltham, MA, USA). Chemiluminescent kit for western blot was from Cyanagen (Bologna, Italy). Ultima gold was from Perkin Elmer (Waltham, MA, USA). 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels, Criterion TGX™ Precast Gels, Turbo Polyvinylidene difluoride (PVDF) Mini-Midi membrane and DC™ protein assay kit were from BioRad (Hercules, CA, USA). High performance thin layer chromatography (HPTLC) and Triton X-100 were from Merk Millipore (Frankfurten, Germany). Polyvinylidene difluoride (PVDF) membrane was from GE Healthcare Life Sciences (Chigago, IL, USA).

Preparation of ganglioside GM1 and its oligosaccharide.

GM1 ganglioside was purified from the total ganglioside mixture extracted from fresh pig brains collected at the slaughterhouse of the Galbani company (Melzo, Italy), according to the procedure developed previously (Tettamanti et al. 1973). The ganglioside mixture, 5 g as sialic acid, was dissolved in prewarmed (36°C) 500 mL of 0.05 M sodium acetate, 1 mM $CaCl_2$ buffer,

pH 5.5. Vibrio cholerae sialidase (1 unit) was added to the solution every 12 h (Acquotti et al. 1994). Incubation at 36°C and under magnetic stirring was maintained for two days, and the solution dialyzed at 23°C for 4 days against 10 L of water changed 5 times a day. The sialidase treated ganglioside mixture was subjected to 150 cm x 2 cm silica gel 100 column chromatography equilibrated and eluted with chloroform-methanol-water, 60:35:5 by vol. The fractions containing GM1, identified by TLC, were pooled, dried and submitted to a further column chromatographic purification using the above experimental conditions. Fractions containing pure GM1 were collected and dried. The residue was dissolved in chloroform-methanol (2:1 v/v) and precipitated by adding 4 volumes of cold acetone. After centrifugation (15.000 x g) the GM1 pellet was separated from the acetone, dried, dissolved in 50 mL of deionized water and lyophilized giving 1350 mg of white powder which was stored at -20°C.

GM1 containing tritium at position 6 of external galactose was prepared by enzymatic oxidation with galactose oxidase followed by reduction with sodium boro[^{3}H]hydride (Sonnino *et al.* 1996). The oligosaccharides II^{3}Neu5Ac-Gg$_4$ and II^{3}Neu5Ac-[^{3}H]Gg$_4$ were prepared by ozonolysis followed by alkaline degradation (Wiegandt and Bucking 1970), from GM1 and [^{3}H]GM1 respectively. Altogether, NMR, MS, HPTLC and autoradiographic analyses showed a homogeneity over 99% for all the prepared gangliosides and oligosaccharides (data not shown).

Tritium labeled and photoactivable GM1, **1** and **2**, and GM1 oligosaccharide, **3**, (Fig. 1), were prepared according to the scheme reported in supplementary materials (Figs. S1, S2 and S3) as previously reported respectively in Mauri *et al.* 2004, Prioni *et al*. 2004 and Chiricozzi *et al*. 2017.

Photoactivable GM1 oligosaccharide, **3**, [6-^{3}H-IVGal-]OligoGM1-N$_3$, bearing the photosensitive group on the glucose, as well as photoactivable GM1 **1**, [6-^{3}H-IVGal-]GM1-N$_3$, bearing the photoactivable group on the fatty acid moiety, were prepared from galactose-tritiated GM1. Photoactivable GM1 **2**, [3-^{3}H-Sph-]GM1-N$_3$ bearing the photoactivable group linked at position 6 of external galactose was prepared from GM1 tritiated at position 3 of the sphingosine.

Briefly, to prepare photoactivable OligoGM1, 52 µmoles of II^{3}Neu5Ac-[^{3}H]Gg$_4$ (0.5 Ci/mmol) were dissolved in 33% ammonia and treated with 1 mg of ammonium hydrogen carbonate. The reaction was maintained under stirring for 48 h at 40°C. The solution was then immediately freeze-dried (Lubineau *et al.* 1995). The same approach was followed for the preparation of

photosensitive GM1 on the external galactose. In this case, however, tritiated GM1 was firstly subjected to enzymatic oxidation at position 6 of the last galactose.

To insert the photoactivable group on fatty acid residue, galactose-tritiated GM1 was firstly submitted to alkaline hydrolysis to remove the stearic acid residue, followed by acid coupling with 12-aminododecanoic acid. The reaction occurred adding 350 µmol of 12-aminododecanoic acid, dissolved in 2.5 mL of dry tetrahydrofuran (12,5%, v/v) to 1.5 mL of dimthylformamide (7,5%) containing 80 µmoles of deacyl-GM1, 1 mL of Triton X-100 (5%, v/v) and 15 mL of dry triethylamine (75%, v/v). The reaction mixture was maintained under continuous magnetic stirring for 24 h at 23°C. The mixture was evaporated under vacuum to 1 mL, and 25 mL of ethyl acetate was added (Sonnino *et al*. 1989).

Finally, all the amino-derivatives were properly treated to insert the chosen photoactivable group. In particular, the azide labeling procedure started with the dissolution of the crude amino-derivatives obtained by previous reaction in 0.5 mL of dry dimethylformamide. Then 1 mg of 2-nitro-4-fluoro-phenylazide and 1 µL of tributylamine in 25 µL of dry DMSO were added under dark conditions. Maintaining dark conditions for the entire process, the reaction mixture was stirred overnight at 80°C. After solvent evaporation, the photoactivable compounds were purified by flash chromatography using chloroform/methanol/2-propanol/water 60 : 35 : 5 : 5 v/v/v/v as eluent for GM1 oligosaccharide (Mauri *et al.* 2004) and chloroform/methanol/water 60 : 35 : 8 v/v/v for GM1 (Sonnino *et al.* 1989). All the derivates were solubilized in methanol and stored at 4°C.

HPTLC analyses were performed using the solvent system chloroform/methanol/0.2% $CaCl_2$, 60:35:8 by vol. Bound sialic acid was determined by the resorcinol-HCl method (Svennerholm 1957; Takki-Luukkainen and Miettinen 1959), pure Neu5Ac being used as the reference standard.

Cell cultures

N2a cells were cultured and propagated as monolayer in DMEM-High Glucose medium supplemented with 10% heat inactivated FBS, 1% L-Glutamine and 1% penicillin/streptomycin, at 37°C in a humidified atmosphere of 95% air / 5% CO_2. Cells were sub-cultured to a fresh culture when growth reached the 80-90% confluence (i.e. every 3-4 days).

Cell authentication

N2a cells are not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee. N2a cells were bought from Sigma-Aldrich to which they were supplied by European Collection of Authenticated Cell Cultures (ECACC) (Catalogue No. 89121404; Lot No. 13K010, passage +9). N2a were used from passage +10 to passage +15 to conduct experiments reported in the present manuscript.

To verify the authentication of employed N2a cells we performed the following tests at the beginning and end of single experimental work.

Morphology check by microscope

To identify the state of cells, we checked cellular morphology by phase contrast microscopy (Olympus BX50 microscope; Olympus, Tokyo, Japan). Morphological outcomes of N2a cells confirmed the expected neuronal/ameboid-like morphology (data not shown).

Growth curve analysis

Cell proliferation was evaluated according to MTT method (Mosmann 1983, Chiaretti *et al.* 2016). Briefly, 2.4 mM MTT (4 mg/ml in PBS) were added to each well and plates were re-incubated for 4 h at 37°C. Medium was carefully removed and replaced with 2-propanol: formic acid, 95:5 (v/v). Plates were gently agitated prior to read the absorbance at 570 nm with a microplate spectrophotometer (Wallac 1420 VICTOR2TM, Perkin Elmer). The growth profile showed a normal growth rate (data not shown).

Mycoplasma detection

Mycoplasma infection was evaluated by fluorescent Hoechst staining (Aureli *et al.* 2012), a fluorescent dye that binds specifically to DNA and that reveals the presence of mycoplasma infections as extracellular particulate or filamentous fluorescence at 400X magnification using NikonEclipse Ni upright microscope. The mycoplasma test has always given negative results (data not shown).

Ganglioside and oligosaccharide treatments

N2a cells were plated at 5 x $10^3/cm^2$ and incubated for 24 h to allow cells attachment and recovery in complete medium before all treatments.

To induce neurodifferentiation, growth medium was removed and cells were pre-incubated in pre-warmed (37°C) Transfectagro™ medium containing 2% FBS, 1% L-glutamine, 1% penicillin/streptomycin, for 30 min at 37°C. Sequentially cells were incubated at 37°C in the presence of 50 μM gangliosides or oligosaccharides (Chiricozzi *et al.* 2017)

Fate of GM1 oligosaccharide photoactivable derivative to N2a cells

Fate of GM1 oligosaccharide photoactivable derivative to N2a cells was determined using tritium-labeled [6-^{3}H-IVGal-]OligoGM1-N_3. After the cell administration with 50 μM [6-^{3}H-IVGal-]OligoGM1-N_3 for different times (3 and 24 h), the medium was removed and the following treatments were performed sequentially: (i) cells were washed five times with 10% FBS-medium to remove the amount of [6-^{3}H-IVGal-]OligoGM1-N_3 weakly associated to the cells – serum removable fraction; (ii) cells were treated with 0.1% trypsin solution to evaluate the [6-^{3}H-IVGal-]OligoGM1-N_3 strongly linked to extracellular domain of plasma membrane proteins – trypsin removable fraction; (iii) cells were lysed in order to evaluate the quantity of [6-^{3}H-IVGal-]OligoGM1-N_3 internalized - trypsin stable fraction. The radioactivity associated to each fraction was determined by liquid scintillation counting using a beta-counter system (PerkinElmer). The entire procedure was performed in dark condition under red safelight omitting cell UV illumination to induce photo-activation. The procedure was established previously to determine the fate of gangliosides administered to cells in culture (Chigorno *et al.* 1985; Chiricozzi *et al.* 2017).

Photolabeling experiments

To study the interplay between GM1 and TrkA, cells were incubated with 50 μM of compound **1**, **2** or **3** (Fig. 1), for 3 h at 37°C in the dark. After incubation, medium was removed and cells were illuminated for 40 min under UV light (λ= 360 nm) on ice to induce photo-activation. All the procedures before exposure to UV light were performed under red safelight. The cells were lysed and subjected to 4-20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on PVDF membrane. Digital autoradiography of the PVDF membrane was performed with Beta-Imager 2000 (Biospace, Paris).The same PVDF membrane was then incubated with anti-TrkA antibody following a blocking step in 5% non-fat milk in TBS-Tween 0,1% (Sonnino *et al.* 1989; Chigorno *et al.* 1990; Sonnino *et al.* 1992; Loberto *et al*. 2003; Chiricozzi *et al.* 2015, Chiricozzi *et al.* 2017).

Isolation of Detergent-Resistant Membrane (DRM) Fractions

To verify the presence of TrkA in the plasma membrane microdomain, N2a cells were incubated in the absence (control) or in the presence of 50 μM GM1 or 50 μM OligoGM1 for 3 h at 37°C.

DRM were prepared by ultracentrifugation on discontinuous sucrose gradient of cells subjected to homogenization with 1% Triton X-100, as previously described (Chiricozzi *et al.* 2015; Schiumarini *et al*. 2017). Briefly, cells were mechanically harvested in PBS 1X and centrifuged at 270 ×g for 10 min at 4°C. Cell pellet was lysed in 1.2 mL of 1% Triton X-100 in TNEV buffer (10mM TrisHCl 10, 150 mM NaCl, 5 mM EDTA pH 7.5) in the presence of 1 mM Na_3VO_4, 1 mM PMSF, and 75 mU/ml aprotinin and homogenized for 11 folds with tight Dounce. Cell lysate (2 mg of cell protein/mL) was centrifuged for 5 min at 1300 ×g at 4°C to remove nuclei and cellular debris and obtain a postnuclear supernatant (PNS). A volume of 1 ml of PNS was mixed with an equal volume of 85% sucrose (w/v) in TNEV buffer containing 1 mM Na_3VO_4, placed at the bottom of a discontinuous sucrose gradient (30-5%), and centrifuged for 17 h at 200,000 ×g at 4°C. After ultracentrifugation, 12 fractions were collected starting from the top of the tube. The light-scattering band, corresponding to the detergent-resistant membrane (DRM) fraction, was located at the interface between 5% and 30% sucrose corresponding to fraction 5 or 6. The entire procedure was performed at 0–4° C on ice immersion. Equal amounts from each fraction were used for protein and lipid analysis.

Protein analysis

Equal amounts of proteins derived from photolabeled cells, from gradient fractions or from GM1/OligoGM1 treated/untreated cells were denatured, separated on 4–20% polyacrylamide gels, and transferred to PVDF membranes using the Trans-Blot® Turbo™ Transfer System (Bio-Rad). The presence of TrkA, Flotillin or Calnexin was determined by specific primary antibody, followed by reaction with secondary HRP-conjugated antibody.

For the time course analysis of TrkA-ERK1/2 activation, proteins derived from GM1/OligoGM1-treated and untreated cells were denatured, separated on 7.5% polyacrylamide gels, and transferred to PVDF membranes. The presence of TrkA, p-TrkA, ERK1/2 and p-ERK1/2 was determined by specific primary antibodies, followed by reaction with secondary HRP-conjugated antibodies.

The data acquisition and analysis were performed using Alliance Uvitec (Cleaver Scientific, United Kingdom).

Protein determination

Protein concentration of samples was assessed using a DC™ protein assay kit according to manufacturer's instructions, using bovine serum albumin as standard.

Lipid extraction and GM1 detection

Sucrose fractions were dialyzed, lyophilized and subjected to lipid extraction. Total lipids were extracted with chloroform/methanol/water, 20 : 10 : 1 by vol, followed by a second extraction with chloroform/methanol, 2 : 1 by vol (Prinetti *et al.* 2011; Chiricozzi *et al.* 2014; Samarani *et al.* 2018). Lipids were separated by Thin Layer Chromatography (TLC), then GM1 ganglioside was specifically detected with HPR-conjugated cholera toxin B (CT-B) staining directly on TLC (Wu and Ledeen 1988, Valaperta *et al.* 2007, Chiricozzi *et al.* 2018a). Briefly, sample were spotted on a silica gel 60 HPTLC plate and developed in chloroform/methanol/0.2% aqueous $CaCl_2$, 50: 42 :11 by vol. Following solvent evaporation, the plate was treated for 30 s with a solution of 0,2% (v/v) polyisobutylmethacrilate in hexane for three times and air dried for 1 h. After 1 h of pre-incubation at 23°C in PBS containing 3% BSA (w/v), the plate was overlaid with CT-B subunit-HPR conjugated (40 ng) in 1% BSA (w/v) at 23°C for 1 h. After washing 3 times with PBS, plates were developed with *o*-phenylendiamine (1 tablet in 1.8 mL of citrate-phosphate buffer 0.05 M (pH 5) and 17 µL of H_2O_2).

Morphological analysis

Cultured cells, treated or not with GM1 or OligoGM1 were observed by phase contrast microscopy (200x magnification, Olympus BX50 microscope; Olympus, Tokyo, Japan). At least 10 fields from each well were photographed for each experiment.

Statistical analysis

Data are expressed as mean ± SEM. The normality of data was not assessed because the number of the samples was too small. Thus, data were analyzed for significance by Mann Whitney test. The analysis was performed with Prism software (GraphPad Software, Inc. La Jolla, CA, USA).

Other analytical methods

NMR spectra were recorded with a Bruker AVANCE-500 spectrometer at a sample temperature of 298 K. NMR spectra were recorded in CDCl3 or CD3OD and calibrate using the TMS signal as internal reference.
Mass spectrometric analysis were performed in positive or negative ESI-MS. MS spectra were recorded on a Thermo Quest Finningan LCQTM DECA ion trap mass spectrometer, equipped with a Finnigan ESI interface; data were processed by Finnigan Xcalibur software system.
All reactions were monitored by TLC on silica gel 60 plates (Merck).
Radioactivity associated with cells and trypsin and serum labile cell fractions was determined by liquid scintillation counting. Digital autoradiography of the PVDF membranes was performed with a Beta-Imager 2000 (Biospace, Paris).

No randomization or blinding procedures were performed for our experiments.
Institutional approval was not needed.
The study was not-preregistered.
No sample calculation was performed to predetermine the sample size.
No test for outliers was conducted because all the data obtained were included in the analysis.
Custom-made materials will be shared upon reasonable request.
The n number (n=) in the figure legend indicates the number of independent cell culture preparations.

Results

Time course of the TrkA-ERK1/2 signaling pathway

The ability of GM1 and its oligosaccharide, OligoGM1, to activate the TrkA-MAPK pathway after 24-48 h from their exogenous administration, has been reported (Chiricozzi *et al.* 2017).

In order to examine the time course of TrkA-ERK1/2 pathway activation induced by GM1 and its oligosaccharide, OligoGM1, the phosphorylation levels of TrkA on Tyr490 and of Erk1/2 on Thr202/Tyr204 were followed by immunoblotting analysis.

As shown in Figure 2, GM1 and OligoGM1 caused a rapid elevation of TrkA phosphorylation, detectable right after 30 min and significantly maintained for over the 12 h time course. The enhancement in ERK1/2 phosphorylation appeared successively, getting significant after 6 h from GM1 and OligoGM1 administration (Fig. 2). The results revealed that, following GM1 and OligoGM1 treatment, TrkA Tyr490 phosphorylation increased two-fold in 3 h and Erk1/2 phosphorylation doubles in 6 h, respect to control untreated cells. Both proteins remained hyperphosphorylated for the following 24-48 h (Chiricozzi *et al.* 2017). In parallel, the morphological analysis revealed the acquisition of neuron-like morphology of N2a cells (sprouting and elongation of neuritis), confirming that the differentiation processes occurred (Fig. 2).

Photolabeling experiments

The ability of GM1 to directly, tightly and specifically associate with TrkA has been demonstrated by Mutoh (Mutoh *et al*. 1995). To characterize in detail which molecular part of GM1 is delegated to the interaction with TrkA receptor, we performed photolabeling experiments using the tritium labeled gangliosides **1** and **2** with the photoactivable group carried by ceramide and external galactose, respectively, and the tritium labeled GM1 oligosaccharide **3** with the photoactivable group carried by glucose. Figure 1 reports the three structures generated in our laboratory according to the scheme described in Figs. S1, S2 and S3 as indicated in supplementary section (Sonnino *et al*. 1989; Mauri *et al* 2004; Prioni *et al.* 2004; Chiricozzi *et al.* 2017).

Previous information reported that under controlled conditions, ganglioside derivatives **1** and **2** administered to the cells are taken up by the cells and incorporated into the cell plasma membrane with metabolic results that are indistinguishable from the endogenous GM1

ganglioside (Mauri *et al* 2004, Prioni *et al.* 2004; Sonnino *et al*. 1989). Differently, the GM1 oligosaccharide derivative **3** does not enter into the cells neither inserts in the plasma membrane, but interacts in a non- covalent manner with the cell surface as shown in Figure S4 (Chiricozzi *et al.* 2017).

N2a cells were incubated for 3 h at 37 °C with the photosensitive derivatives, under dark conditions, and illuminated by UV-light for 40 min at 4°C to induce covalent cross-linking between radioactive derivatives and neighboring cell components. Under UV irradiation. the azide of the nitrophenyl-azide group becomes a nitrene, that immediately generates covalent bonds with adjacent molecules, due to its high reactivity. In this way, proteins interacting with the azide bearing radioactive molecules becomes radiolabeled as well (Sonnino *et al.* 1989). Cell proteins were separated by SDS-PAGE, transferred on a PVDF membrane and visualized by digital autoradiography as well as by colorimetric assay. Among the entire N2a protein pattern (Fig. S5, line 2), only few radioactive bands were detected on PVDF (Fig. S5, line 1). The radioactive tracks identify proteins cross-linked with the photoactivated compound, therefore proteins that were in close proximity to photoactivable compound before illumination. A radioactive protein pattern was identified using both the ganglioside derivatives **1** and **2** incorporated into the plasma membranes, as well as using the oligosaccharide derivative **3** that was not taken up by the cells. In the three experimental models, the difference between the protein and the radioactive protein patterns suggests specific protein-ganglioside and protein-oligosaccharide interactions (Fig. 3).

Following the autoradiography, the PVDF membranes were immunostained with anti-TrkA antibody. The band corresponding to TrkA signal was found to perfectly overlap a radiolabeled band at 140 kDa in cells treated with ganglioside derivative **2**, carrying the photoactivable group on the external galactose and with the oligosaccharide derivative **3**, carrying the photoactivable group on the glucose (Fig. 3B-C). No correspondence of TrkA signal with radioactive track was found when cells were treated with the GM1 derivative **1**, carrying the photoactivable group at the end of ceramide (Fig. 3A).

These results suggest that the TrkA-GM1 complex formed through the interaction between the TrkA and the oligosaccharide head of the ganglioside GM1, does not involve the presence of ceramide portion in close proximity to the receptor.

Membrane lipid domains characterization

The above results support the hypothesis that TrkA and GM1 inserted into the external lipid layer of the plasma membrane, are near enough to interact. Contrary to this possibility, there are previous information reporting that the ganglioside GM1 localizes in lipid membrane domains, the lipid rafts, and that the TrkA receptor places in the fluid membrane environment separated from the lipid rafts (Limpert *et al.* 2007; Ichikawa *et al.* 2009; Pryor *et al.* 2012).

To better understand this point, after treating N2a cells with GM1 ganglioside or with its oligosaccharide, lipid rafts were isolated as the detergent-resistant membrane (DRM), according to the procedure described in Methods section.

Lipids were extracted from each fraction, separated by HPTLC and ganglioside GM1 content was revealed by TLC-staining with cholera toxin B subunit (CT-B). In all experimental conditions (control cells, GM1-treated cells and GM1 oligosaccharide treated cells) GM1 was found in the rigid membrane fractions non-solubilized by the detergent (Fig. 4), which is in total agreement with the large information available from the literature (Limpert *et al.* 2007; Ichikawa *et al.* 2009; Pryor *et al.* 2012). Differently, in any experimental condition, western blotting analysis revealed that TrkA was present in the fluid membrane fraction, solubilized by the detergent (Fig. 4). This result supports the photoactivable data and highlights that TrkA and GM1 interacts through the oligosaccharide chain in the extracellular environment since GM1 and TrkA belong to separate membrane portions.

Discussion

Gangliosides are amphiphilic compounds with a good balance between the hydrophilic head, represented by the oligosaccharide group, and the highly hydrophobic ceramide (Sandhoff *et al.* 2018). The gangliosides oligosaccharide protrudes into the extracellular environment where may interact with the extracellular part of the other membrane components, while the ceramide is inserted into the lipid membrane layer serving to anchor the total structure in restricted membrane areas known as “lipid rafts” (Sonnino *et al.* 2006, 2017). It is believed that in the lipid rafts, sphingolipids modulate the functional features of several membrane proteins (Coskun and Simons 2011). Since gangliosides are highly segregated together with cholesterol in liquid-ordered lipid domains with locally reduced fluidity (Sonnino *et al.* 2006, Grassi *et al.* 2018), it is suggested that proteins co-segregating with them display a restricted lateral motility. Such microenvironment, characterized by stable interactions results instrumental for ganglioside-dependent protein specific fine modulation. In addition, glycosphingolipid oligosaccharide chains are recognized by soluble ligands, such as that of GM1 by cholera toxin (Svennerholm 1976) and that of neutral glycolipid Gb_3Cer by verotoxin (Lingwood 1999; Chiricozzi *et al.* 2018b). Moreover, the GD1a and GT1b oligosaccharide chains interact with the myelin-associated glycoprotein (Schnaar and Lopez 2009) belonging to the interfacing membrane of adjacent cells.

However, in most cases the molecular mechanisms underlying the modulatory effect of gangliosides on the protein functions remains obscure.

The increase of GM1 at the cell surface, obtained with any experimental strategy, promotes neurite production and cell differentiation in neuroblastoma cells (Facci *et al.* 1984; Farooqui *et al.* 1997; Mutoh *et al.* 2002; Rodriguez *et al.* 2001; Da Silva *et al.* 2005; Valaperta *et al.* 2007; Schneider *et al.* 2015). Experimental results indicate that this is due to TrkA activation by GM1 (Rabin and Mocchetti 1995; Ferrari *et al.* 1995; Farooqui *et al.* 1997; Mutoh *et al*. 1995, 2002; Rabin *et al.* 2002; Duchemin *et al.* 2002) and more in detail, due to the interaction between TrkA and the head group of GM1, its oligosaccharide (Chiricozzi *et al.* 2017). In PC12 cells, where GM1 in the absence of NGF is not able to induce neurodifferentiation (Ferrari *et al.* 1983), the GM1 association with TrkA strongly improves neurite outgrowth and neurofilament protein expression elicited by a low dose of NGF, inducing a 3 fold increase in NGF-induced Trk autophosphorylation (Mutoh *et al.* 1995). Important to note, that NGF, even if not synthetized

by N2a cells (Leon *et al.* 1994) is present in our experimental condition since the culture medium used contains serum (Chiricozzi *et al*. 2017). Molecular dynamics calculations suggest that hydrogen bonds and ionic interaction between sugars and TrkA-NGF amino acids stabilize a TrkA-NGF-GM1 complex (Chiricozzi *et al.* 2017). This means that the ganglioside oligosaccharide is not more distant than 4-5 Å from the receptor, during the interaction. GM1 belongs to a rigid membrane environment and is considered one of the marker of lipid rafts. On the other hand, it is reported that TrkA is associated to a membrane fluid environment displaying, at least under specific conditions, some dynamics (Limpert *et al.* 2007; Ichikawa *et al.* 2009; Pryor *et al.* 2012). Here, we fractionated the total cell homogenate prepared from cells treated, or not treated, with ganglioside GM1 or its oligosaccharide and, in agreement with the available information, GM1 was found in the rigid membrane fractions while TrkA was found in the fluid fraction solubilized by the detergent (Fig. 4).

From our previous results, we know that the ceramide portion of GM1 is not necessary for the TrkA activation (Chiricozzi *et al.* 2017), and the results on the membrane topology of TrkA and GM1 suggest that GM1 ceramide is not strictly part of the environment of TrkA. To better understand how the TrkA-GM1 interaction occurs, we synthesized three tritium labeled photoactivable compounds (Fig. 1): **1)** GM1 carrying the photoactivable group linked at the end of the acyl moiety, **2)** GM1 carrying the photoactivable group linked to the external galactose, and, **3)** the free GM1 oligosaccharide with the photoactivable group linked to glucose.

The three compounds were added to the cell medium. We know that, under established and here used conditions, the GM1 derivative administered to the cells is taken up by the cells becoming component of the cell plasma membranes and metabolically indistinguishable from the endogenous GM1 (Sonnino *et al.* 1989). On the contrary, the GM1 oligosaccharide is not taken up by the cells (Fig. 4S).

Cells were UV illuminated and proteins analyzed for their tritium content, mirror of the stable cross linkage of the proteins with the photoactivated compound (Fig. 3). TrkA resulted cross linked to the GM1 oligosaccharide and to the GM1 carrying the photoactivated group on the position 6 of external galactose, but not with GM1 carrying the photoactivable group at the end of the acyl chain and located in the middle of membrane double layer. This result suggests that the distance between the GM1 ceramide moiety and the transmembrane portion of TrkA is too wide to establish the covalent linkage. This in agreement with the result indicating that TrkA is detergent soluble, while GM1 is not (Fig. 4).

It is reported that, at least keeping precise conditions, TrkA, that shows high dynamics within the membrane components, moves from fluid to rigid environments (Limpert *et al.* 2007; Ichikawa *et al.* 2009; Pryor *et al.* 2012). According to this, we should consider that the administration of GM1 to the cells, and its incorporation into the membrane rigid domain (Facci *et al.* 1984; Limpert *et al.* 2007; Ichikawa *et al.* 2009; Pryor *et al.* 2012), allows to reach a GM1 membrane concentration high enough to block the dynamics of TrkA, forming then the TrkA-GM1 complex through a TrkA-GM1 oligosaccharide interaction. Thus, when the TrkA approaches the boarder of the lipid rafts due to its dynamics in the fluid membrane, the extracellular environment of the soluble portion of TrkA, with an extension of the order of several tens of Å, may reach the GM1 oligosaccharide with no necessity for TrkA to belong to the same lipid membrane domain of GM1.

Acknowledgments and conflict of interest disclosure

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Online supplemental material.

Figure 1S. This figure shows the scheme of the synthetic process for the preparation of [Gal-6-3H]GM1(Cer-N3)

Figure 2S. This figure shows the scheme of the synthetic process for the preparation of [Sph-3-3H]GM1(Gal-N3)

Figure 3S. This figure shows the scheme of the synthetic process for the preparation of [Gal-6-3H]OligoGM1(Glc-N3).

Figure 4S. This figure shows the association of GM1 oligosaccharide photoactivable derivative to N2a cells

Figure 5S. This figure shows the N2a protein patterns

Figure 6S. This figure shows the experiment time line diagrams.

Figure Legends

Figure 1 – Chemical structure of GM1 photoactivable derivatives. A) [Gal-6-^{3}H]GM1(Cer-N_3): tritiated GM1 derivative with a nitrophenylazide at the end of acyl chain; **B)** [Sph-3-^{3}H]GM1(Gal-N_3)]: tritiated GM1 derivative with the nitrophenylazide at position 6 of external galactose; **C)** [Gal-6-^{3}H]OligoGM1(Glc-N_3): tritiated GM1 oligosaccharide derivative with the nitrophenylazide linked to the position 1 of glucose.

Figure 2 – Time course of TrkA and ERK1/2 pathway activation followed by N2a differentiation. A-B) N2a cells were treated with 50 μM GM1 or OligoGM1 up to 12 h. The figure shows the expression of TrkA, phosphorylated TrkA (Tyr490), ERK1/2 and phosphorylated ERK1/2 in cell lysate by means of specific antibodies and reveled by enhanced chemiluminescence. Top: immunoblotting images are shown. Bottom: Semi-quantitative analysis of phosphorylated TrkA (A) and ERK1/2 (B) related to total level of TrkA and ERK1/2, respectively. Data are expressed as fold increase over control of the mean ± SEM from three independent cell culture preparations (*p < 0.05, Mann Whitney test, n=3). **C)** Morphological analysis of N2a cells. 1, control; 2, OligoGM1. Following 24 h incubation, cells were analyzed by contrast phase microscopy with 200x magnification. Images are representative of ten independent cell culture preparations (n=10) [white square: control, light gray square: GM1; dark gray square: OligoGM1].

Figure 3 – Interaction between TrkA and GM1 in N2a cells. Photoactivable derivatives of GM1 were administered to N2a cells and cells were then illuminated. Proteins of cell lysate were submitted to 4-10% acrylamide SDS-PAGE, transferred on PVDF membrane and visualized by digital autoradiography for 96 h (right). The same PVDF was blotted using specific antibody anti TrkA (left). The images are representative of three independent cell culture preparations. **A)** Interaction between TrkA and [Gal-6-^{3}H]GM1(Cer-N_3)M; **B)** Interaction between TrkA and [Sph-3-^{3}H]GM1(Gal-N_3)]; **C)** Interaction between TrkA and [Gal-6-^{3}H]OligoGM1(Glc-N_3); **B)** Glycoconjugates representation is according to Varki *et al*. 2015 (green star: photoactivable group).

Figure 4 – Sucrose gradient fractions characterization. N2a cells were incubated in the absence (**A**, control), or in the presence of 50 µM GM1 (**B**) or 50 µM OligoGM1 (**C**) for 3 h at 37°C. Cells were subsequently subjected to sucrose gradient ultracentrifugation to prepare plasma membrane microdomains. Twelve fractions were collected from the top of the tube, with fractions 4-6 corresponding to the detergent resistant membrane (DRM) fractions and fractions 10-12 corresponding to high density (HD) fractions. *Left:* Immunoblotting against the TrkA, calnexin (HD marker) and flotillin (DRM marker) performed on postnuclear supernatant (PNS) and on the 12 fractions. Images are representative of three independent cell culture preparations.. *Right:* GM1 detection among total lipids extracted from each sucrose fraction, revealed by cholera toxin subunit B (CT-B) TLC-staining (std: GM1 standard).

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