UNIVERSITÀ DEGLI STUDI DI MILANO

Dottorato di Ricerca in Scienze Biochimiche
XXXII ciclo

Dipartimento di Biotecnologie Mediche
e Medicina Traslazionale

GM1 oligosaccharide accounts for GM1 role in enhancing neuronal development acting on TrkA-MAPK pathway

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Anno Accademico 2018/2019

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Abstract

Abstract

The GM1 ganglioside is a mono-sialylated glycosphingolipid present in the outer layer of the cell plasma membrane and abundant in neurons. Numerous *in vitro* and *in vivo* studies highlight the role of GM1 not only as a structural component but also as a functional regulator. Indeed, GM1 enrichment in membrane microdomains promotes neuronal differentiation and protection, and the GM1 content is essential for neuronal survival and maintenance.

Despite many lines of evidence on the GM1-mediated neuronotrophic effects, our knowledge on the underlying mechanism of action is scant. Recently, the oligosaccharide chain of GM1 (oligoGM1) has been identified as responsible for the neuritogenic properties of the GM1 ganglioside in neuroblastoma cells. The oligoGM1-mediated effects depend on its binding to the NGF specific receptor TrkA, thus resulting in the TrkA-MAPK pathway activation.

In this context, my PhD work aimed to confirm the role of the oligoGM1, as the bioactive portion of the entire GM1 ganglioside, capable of enhancing the differentiation and maturation processes of mouse cerebellar granule neurons.

First, we performed time course morphological analyses on mouse primary neurons plated in the presence or absence of exogenously administered gangliosides GM1 or GD1a (direct GM1 catabolic precursor). We found that both gangliosides increased neuron clustering and arborization, however only oligoGM1 and not oligoGD1a induced the same effects in prompting neuron migration. This result suggests the importance of the specific GM1 saccharide structure in mediating neuronotrophic effects. Then we characterized biochemically the oligoGM1-mediated effect in mouse primary neurons, and we observed a higher phosphorylation rate of FAK and Src proteins which are the intracellular key regulators of neuronal motility. Moreover, in the presence of oligoGM1 cerebellar granule neurons showed increased level of specific neuronal markers (e.g., β3-Tubulin, Tau, Neuroglycan C, Synapsin), suggesting an advanced stage of maturation compared to controls. In addition, we found that the oligoGM1 accelerates the expression of the typical ganglioside pattern of mature neurons which is characterized by high levels of complex gangliosides (i.e., GM1, GD1a, GD1b, and GT1b) and low level of the simplest one, the GM3 ganglioside.

To study the mechanism of action of the oligoGM1, we used its tritium labeled derivative and we found that the oligoGM1 interacts with the cell surface without entering the cells. This finding suggests the presence of a biological target at the neuronal plasma membrane.

Interestingly, we observed the TrkA-MAP kinase pathway activation as an early event underlying oligoGM1 effects in neurons.

Our data reveal that the effects of GM1 ganglioside on neuronal differentiation and maturation are mediated by its oligosaccharide portion. Indeed, oligoGM1 interacts with the cell surface, thus triggering the activation of intracellular biochemical pathways that are responsible for neuronal migration, dendrites emission and axon growth.

Overall, our results point out the importance of oligoGM1 as a new promising neurotrophic player.

Abbreviations:

BSA: bovine serum albumine

CGN: cerebellar granule neurons

CMP: cytidine-5′-monophosphate

CNS: central nervous system

Ctx-B: B subunit of cholera toxin

ERK1/2: extracellular signal-regulated protein kinases 1 and 2

FAK: focal adhesion kinase

FBS: fetal bovine serum

GDNF glial-derived growth factor

Hp-TLC: high performance thin layer chromatography

HRP: horse raddish peroxidase

MAPK: mitogen activated protein kinase

NCX: Na/Ca exchanger

NGF: nerve growth factor

OligoGD1a: GD1a-oligosaccharide

OligoGM1: GM1-oligosaccharide

PBS: phosphate saline buffer

PD: Parkinson's Disease

PNS: peripheric nervous system

PVDF: polyvinylidene difluoride

SDS-PAGE: sodium-dodecyl-sulphate-polyacrylamide gel electrophoresis

Trk: tropomyosin receptor kinase

TRPC5: Transient receptor potential channel 5

UDP: uridine diphosphate

Introduction

1. GM1 structure, nomenclature and structural properties

GM1 belongs to the family of glycosphingolipids containing at least one sialic acid residue (neuronaminic acid, Neu), i.e. gangliosides. In particular, GM1 contains a single residue of sialic acid and belongs to the ganglio- tetrahexosyl series, with formula β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)-]-β-Gal-(1-4)-Glc-(1-1)-Ceramide, represented in Figure I1 (panel A).

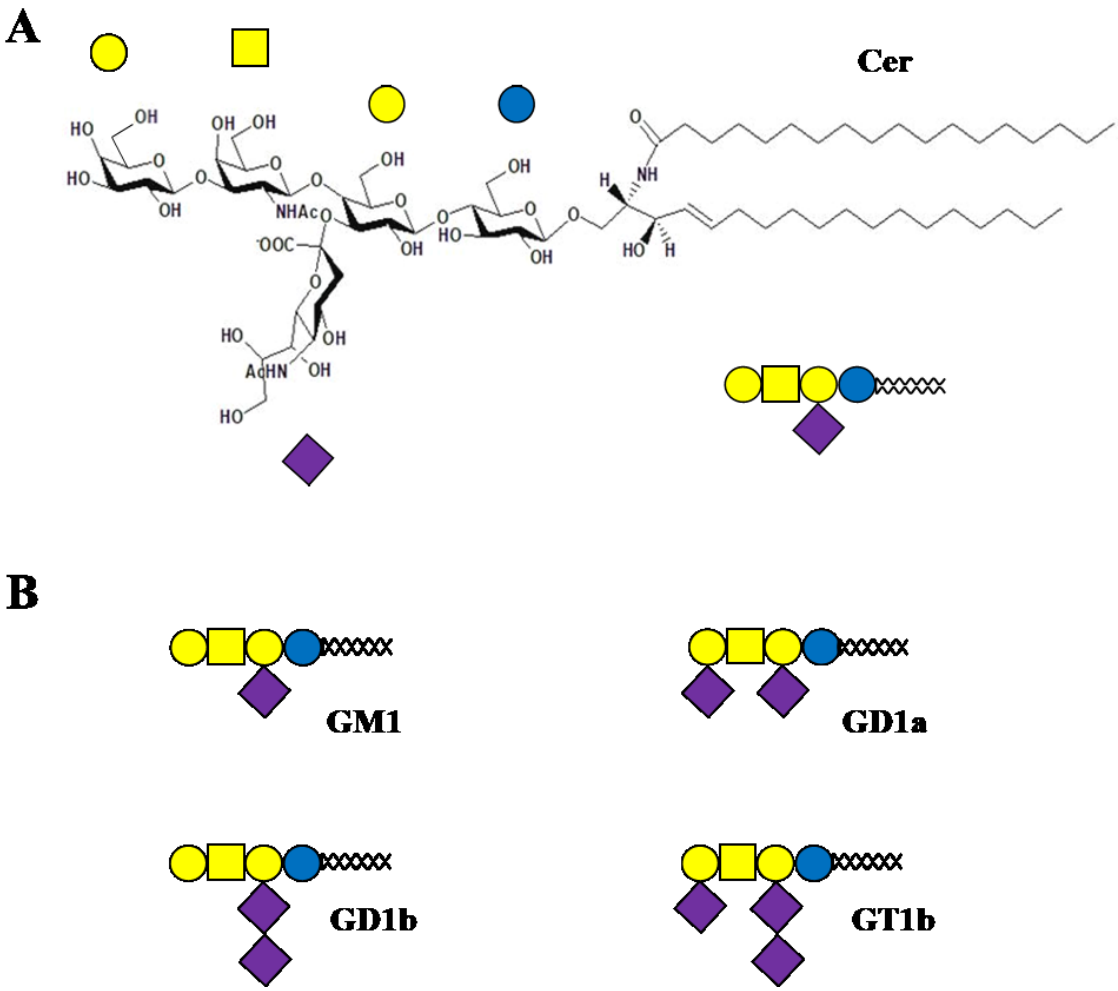


Figure I1. Schematic representation of gangliosides. A: GM1 ganglioside consists of neutral core structure Galβ3-GalNAcβ4-Galβ4 -Glcβ1-Cer and one N-acetylneuraminic acid attached to the inner galactose. B: Schematic representation of major gangliosides in vertebrate brain: GM1, GD1a, GD1b and GT1b. Blu circle = Glucose. Yellow circle = Galactose. Yellow square = GalNAc. Purple rhombus = Neu5Ac.

Several Neu structures have been discovered and in humans the most common is 5-N-acetyl derivative (Neu5Ac or NANA) (Schauer *et al*. 1982).

The specific names for the gangliosides provide information about their structure, according to International Union of Pure and Applied Chemistry (IUPAC)- International Union of Biochemistry (IUB) nomenclature (Chester 1998). The capital letter G refers to ganglioside, and the subscript M indicates that the molecule contains one unit of sialic acid. The numbered subscript refers to the carbohydrate sequence that is attached to the ceramide. In particular, 1 stands for Gg_4 neutral series, i.e. β-Gal-(1-3)-β-GalNAc-(1-4)-β-Gal-(1-4)-Glc oligosaccharide structure (Aureli *et al.* 2016).

Like all sphingolipids, the hydrophobic portion of GM1 is represented by the ceramide. There are different types of ceramides in nature and generally consist of a basic long hydrophobic chain base, which is called sphingosine (Sph) attached to a fatty acid by amidic bond. Within the brain, sphingosine may have 18 or 20 carbon atoms (C18-C20) at different ratios in nervous system (Karlsson 1970) with C20-Sph barely detectable in the fetus but becoming the most represented sphingosine in adult human brain (Sonnino and Chigorno, 2000). Regarding the fatty acid of ceramide, stearic acid accounts for 90% of that present in the nervous system.

In biological environments, GM1 has a net negative charge due to the presence of sialic acid. The presence of the pentasaccharidic portion confers generally a high degree of hydrophilicity, both for sialic acid and for the intrinsic hydrophilicity of sugars. The combination of the hydrophobic lipid portion and the hydrophilic oligosaccharide portion gives the GM1 molecule an amphiphilic character that characterizes its chemical-physical behaviors but also the biological ones.

In aqueous solution, GM1 is soluble and tends to form ellipsoidal micellar aggregates due to the hydrophobic effect (Cantù *et al.* 1986). GM1 micelles form at very low concentration. In fact, the minimum micellar concentration of GM1 is 10^{-8}-10^{-9} M to indicate that regardless of its concentration, in aqueous solution, the concentration of GM1 monomers cannot exceed that of minimum micellar concentration (Sonnino *et al*. 1994; Corti *et al*. 1980; Ulrich-Bott and Wiegandt 1984).

Aggregative properties are of significant relevance when gangliosides are exogenously administered to cells. In fact, only the monomeric GM1 becomes part of the plasma membrane of the cells, while the micelles interact with the plasma membrane and are internalized by endocytosis, to be then metabolized in the endo-lysosomal system (Valsecchi *et al*. 1992; Saqr *et al*. 1993).

GM1, like all gangliosides, is stably inserted into the outer layer of the plasma membrane with the portion of the ceramide inserted into the lipid bilayer which interacts *via* hydrophobic interactions with the surrounding lipids. The oligosaccharide is exposed in the extracellular aqueous environment and its conformation has been studied in detail by means of nuclear magnetic resonance (NMR) experiments and molecular modeling (Acquotti *et al*. 1990; Brocca *et al*. 1998; 2000; Fantini and Yahi 2015). The bonds between the Gal-GalNAc and Gal-Glc disaccharides are more dynamic with 2 possible conformations, while the internal trisaccharide GalNAc-(NeuAc)-Gal is considered as a rigid block with one possible conformation. The link between Glc and ceramide suggests that the entire oligosaccharide head of GM1 is placed perpendicular to the plasma membrane (Acquotti *et al*. 1990; Brocca *et al.* 2000).

The GM1 monomers are then distributed in membrane as a group of conformers giving high dynamism to the structure. In this way the monomers can interact, through the lipid component and the oligosaccharide component, both with the membrane proteins and with each other, segregating and clustering in the membrane (Brocca *et al.* 1998; Mauri *et al.* 2012).

To note, crystallographic studies have been useful to visualize three-dimensional structures of the GM1 pentasaccharide recognized by cholera toxin, AB_5 toxin, and polyoma virus (Holmgren *et al*. 1975; Neu *et al.* 2008; Nagatsuka *et al*. 2013) In these structures, the outer branch of the carbohydrate chain is involved in the interactions with these hazardous binders. The pentasaccharide conformations differ among these complexes and are deviated from the NMR structures, suggesting that GM1 glycan undergoes conformational alteration upon binding to proteins in a ligand-dependent manner (Yagi-Utsumi and Kato 2015).

2. GM1 metabolism and topology

Gangliosides and therefore GM1 are particularly enriched in the nervous system cells. They abound especially in the membranes of neurons but have also been identified in glial cells and GM1 has also been found in astrocytes, oligodendrocytes and microglia.

De novo biosynthesis of GM1, as for all gangliosides, occurs inside the cells, at the level of Golgi compartment, the intracellular organelle most enriched in glycosphingolipids. In the Golgi the assembly of the oligosaccharide head occurs by the sequential adding of the individual activated saccharide units by the action of glycolipid glycosyltransferases, a pathway primarily identified by Roseman in 1970 (Roseman *et al*. 1970).

As shown in Figure I2, complex gangliosides are stepwise synthesized from simpler ones. The first ganglioside, GM3, is produced from lactosylceramide by cytidine-5′-monophosphate (CMP)-sialic acid:LacCer α2-3 sialyltransferase (ST-I), which adds an α2, 3-linked sialic acid to lactosylceramide. Further addition of sialic acids by CMP-sialic acid:GM3 α2-8 sialyltransferase (ST-II) and CMP-sialic acid:GD3 α2-8 sialyltransferase generates GD3 and GT3, respectively. These simple gangliosides constitute the complex gangliosides belonging to the a-, b-, and c-series, respectively. Elaboration of the ganglioside structures is achieved by other specific glycosyltransferases, such as UDP-GalNAc:LacCer/GM3/GD3/GT3 β1-4 N-acetylgalactosaminyltransferase (GalNAcT), UDP-Gal:GA2/GM2/GD2/GT2 β1-3 galactosyltransferase, CMP-sialic acid:GA1/GM1/GD1b/GT1c α2-3 sialyltransferase, and CMP-sialic acid:GM1b/GD1a/GT1b/GD3 α2-8 sialyltransferase (Svennerholm *et al*. 1994; Yu *et al*. 2004; 2008).

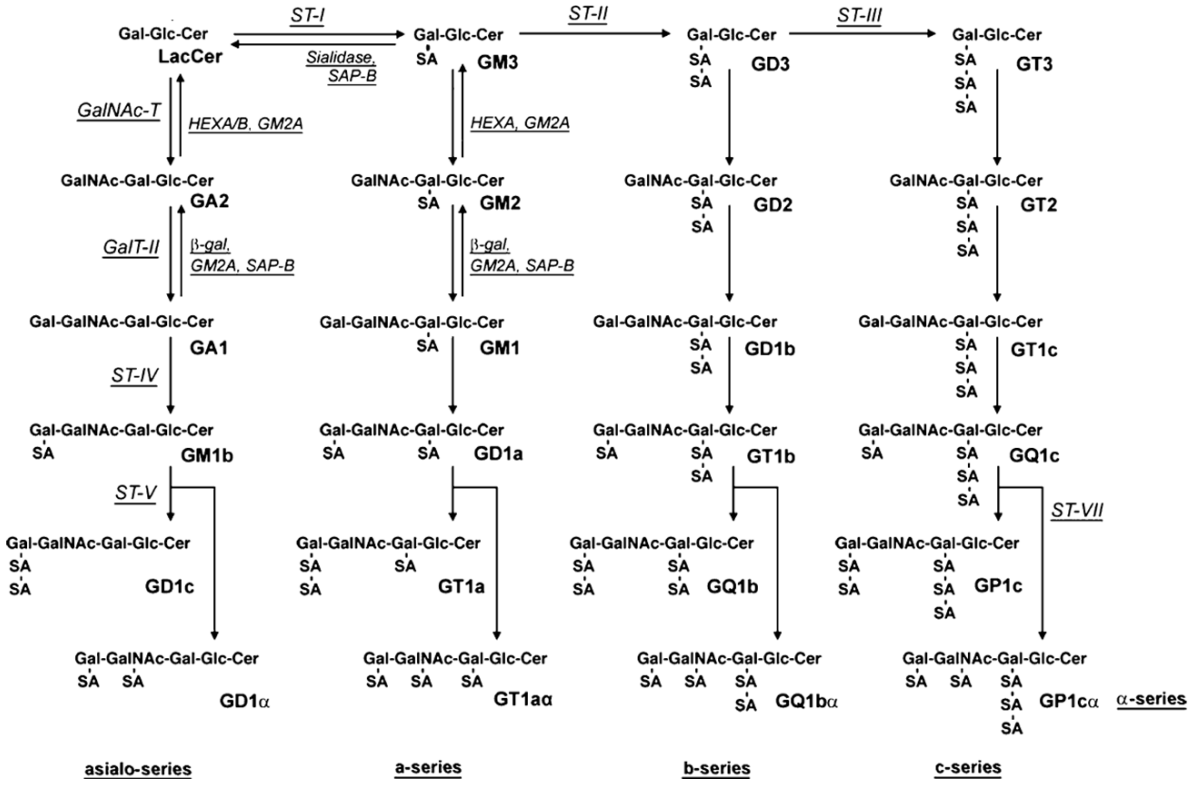


Figure I2: Structure and metabolic pathways of gangliosides. β-gal, lysosomal acid β-galactosidase; Cer, ceramide; GALC, galactosylceramidase; GalNAc-T, N-acetylgalactosaminyltransferase I (GA2/GM2/GD2/GT2-synthase); GalT-I, galactosyltransferase I (lactosylceramidesynthase); GalT-II, galactosyltransferase II (GA1/GM1/GD1b/GT1c-synthase); GalT-III, galactosyltransferase III (galactosylceramidesynthase); GLCC, glucosylceramidase; GlcT

glucosyltransferase (glucosylceramide synthase); GM2A, GM2 activator protein; HEX, b-N-acetylhexosaminidase; SA, sialic acid; SAP, saposin; ST-I, sialyltransferase I (GM3-synthase); ST-II, sialyltransferase II (GD3-synthase); ST-III, sialyltransferase III (GT3-synthase); ST-IV; sialyltransferase IV (GM1b/GD1a/GT1b/GQ1c-synthase); ST-V, sialyltransferase V (GD1c/GT1a/GQ1b/GP1c-synthase); ST-VII, sialyltransferase VII (GD1a/GT1aa/GQ1ba/GP1ca-synthase). In this Figure, the nomenclature of gangliosides is based on that of Svennerholm (Svennerholm 1963).

During the normal turnover of plasma membranes, GM1 is degraded at the level of the endo-lysosomal compartment, more precisely within the intralysosomal vesicles as revealed by immunoelectron microscopy studies (Fürst *et al*. 1992; Möbius *et al*. 1999). As for the synthetic pathway, the degradation process occurs in a stepwise manner, mainly by water-soluble but often protein and membrane associated hydrolases with the help of lipid binding proteins, SAP (Kolter *et al*. 2005).

In this way a first action of the β-galactosidase on GM1 forms the GM2 which becomes substrate of the β-hexosaminidase to give GM3. The action of lysosomal sialidases, mainly Neu1 and Neu4, generates lactosylceramide, which after hydrolysis by galactosidase and glucosylcerebrosidase leads to the formation of ceramide which can be further degraded or recycled.

It is important to note that the genetic alteration of one of the enzymes involved in the metabolism of GM1 and other glicosphingolipids is associated with several cellular functional anomalies leading to severe pathological conditions (Breiden and Sandhoff 2018).

Studies with genetically modified mice by disruption of ganglioside synthetic enzymes allowed to investigate the role of each molecular specie (Sheikh *et al*. 1999; Proia 2003; Furukawa *et al*. 2004). These include the much-studied *β4galnT1*$^{-/-}$ and *β4galnT*$^{+/-}$ mice null or heterozygous for GM2/GD2 synthase, which have provided key clues on the functions of gangliosides and especially GM1 (Furukawa *et al*. 2002, 2004; Schengrund 2015).

On the other hand, the alteration of the enzymes involved in the catabolism of gangliosides are associated with pathological syndromes belonging to gangliosidosis family (Sandhoff 2013).

GM1 is mainly represented on the neuronal plasma membrane (Tettamanti *et al*. 1973), where initially, thanks to studies using ligands such as B subunit of Cholera toxin and anti-GM1 antibodies with high-affinity for GM1, an enrichment of GM1 was observed

exclusively at presynaptic and postsynaptic membranes of nerve endings (Hansson *et al*. 1977; Ledeen *et al*. 1978). Subsequent studies have revealed the presence of GM1 and other gangliosides over large portions of the neuron comprising the axolemma and the soma, although their concentration was found to be lower in perikaryon and higher in synaptic terminals (Devries *et al*. 1980). This non-homogeneous distribution of gangliosides is due to the rapid vesicular axonal transport to nerve endings (Forman *et al*. 1972) and, in parallel, to retrograde transport from terminations to the neuronal soma, to be re-used or metabolically processed (Aquino *et al*. 1987).

In the plasma membrane, GM1 is distributed and segregated in lipid microdomains which are called lipid rafts. From an operational point of view, they are known as detergent resistant membrane that are isolated as low-density fractions by density gradient ultracentrifugation. These microdomains are enriched in gangliosides, sphingomyelin, cholesterol, phosphatidylinositol but are also populated by lipid-binding proteins (Simons and Sampaio, 2011). Although they are considered lipid raft markers, actually gangliosides represented only 6- 7% of the lipids present in microdomains of primary neurons, with more than 50% consisting of glycerophospholipids (Sonnino *et al*. 2007). Furthermore, GM1 is also the less abundant with GD1a, GD1b and GT1b representing the majority of gangliosides in mature neurons (structures in Figure I1, panel B). However, GD1a is considered as a reserve pool of GM1 which is easily converted into GM1 by membrane endogenous sialidase Neu3 (Sonnino *et al*. 2011; Miyagi *et al*. 2012).

In fact, another important site of gangliosides metabolism in addition to the endo-lysosomal system is represented by the plasma membrane, which hosts glycohydrolases and sialidases for the local surface and fine remodeling of the glycolipid composition (Aureli *et al*. 2014).

Membrane microdomains represent important cellular signaling platforms through which cells receive signals from outside and initiate reactions that involve cellular responses and changes. Despite their paucity, gangliosides are recognized as fundamental elements with an active role not only for the formation, stabilization and dynamics of microdomains (Sonnino *et al*. 2007; Ohmi *et al*. 2012), but also because they represent primary players in initiating and regulating the signaling pathways. This is particularly true for GM1, whose levels are finely regulated during the processes of differentiation and neuronal survival (addressed in the section: *Functional role of GM1 in the Nervous System*).

In addition to the plasma membrane, small amounts of gangliosides have also been found in the membrane of the mitochondria, nucleus and in the endoplasmic reticulum, but little is known about their origin and metabolism in these organelles (Breiden and Sandhoff 2018). GM1 and GD1a were found in the nuclear envelope along with Neu3 sialidase (Ledeen *et al.* 2011). Here the GM1 plays an important role in regulating the activity of the Na/Ca exchanger (NCX) and thus contributing to the modulation of nuclear calcium levels (Xie *et al.* 2002).

3. Functional role of GM1 in the Nervous System

The central and peripheral nervous tissues are the sites most abundant in gangliosides (Ledeen and Wu 2018). In the brain, gangliosides concentrate in the gray substance with respect to white matter, indicating that neurons are the main site of gangliosides (Svennerholm *et al*. 1994). As mentioned above, gangliosides are involved in a wide range of biological processes in addition to their role as simple structural membrane components. The content and composition of gangliosides change significantly during differentiation, aging and neuronal damage (Ngamukote *et al*. 2007; Yu *et al*. 2009). Among all the gangliosides, GM1 is surely the most studied for its neurotrophic and neurorestorative properties (Aurelie *et al*. 2016; Ledeen and Wu 2015). Its functions have been discovered through *in vivo* studies, with the use of genetically modified animal models with alterations in the biosynthetic pathways of gangliosides and *in vitro* using cultures of neuronal-like cells or primary neurons (Ledeen and Wu 2015, 2018).

The physiological but also pharmacological properties of GM1 have been studied using mainly two approaches: through the manipulation of endogenous GM1 and through the application of exogenous GM1 (Ledeen and Wu 2015, 2018; Schengrund 2015). The first approach involves the use of endogenous sialidase which locally modifies the ganglioside composition of plasma membrane microdomains, reducing the polysialogangliosides in favor of the GM1 increase. The administration of the B subunit of cholera toxin (Ctx-B) or GM1-specific polyvalent antibodies (of the IgM series), alone or combined, as cross-linking agents, was performed in many studies to obtain the clustering of endogenous GM1 molecules and concomitantly of proteins containing ganglioside binding domains, to modulate the activation of downstream pathways.

On the other hand, the exogenous administration of GM1 or its derivatives modified due to the presence of radioisotopes or photoactivable groups, allowed to study the metabolism of ganglioside and the potential lipid-lipid and lipid-proteins interactions among adjacent molecules.

Both approaches have been useful in defining the multiple properties of GM1, which has recently been defined as a *true factotum* of nature (Ledeen and Wu 2015), with results that are often consistent but sometimes conflicting given the important differences between the two experimental approaches.

Decades of studies have brought to light ever-increasing evidence of the role of GM1 in neuronal differentiation and maturation, in survival and in mechanisms of protection and repair of nerve cells.

3.1. GM1 in neuronal differentiation and early development

It has been well known that ganglioside composition of the nervous system undergoes remarkable changes during development. The ganglioside content in the brain drastically increases as development proceeds and simultaneously the expression pattern of gangliosides shifts from simple gangliosides, such as GM3 and GD3, to complex gangliosides, such as GM1, GD1a, GD1b and GT1b in rodents and humans as well (Figure I3).

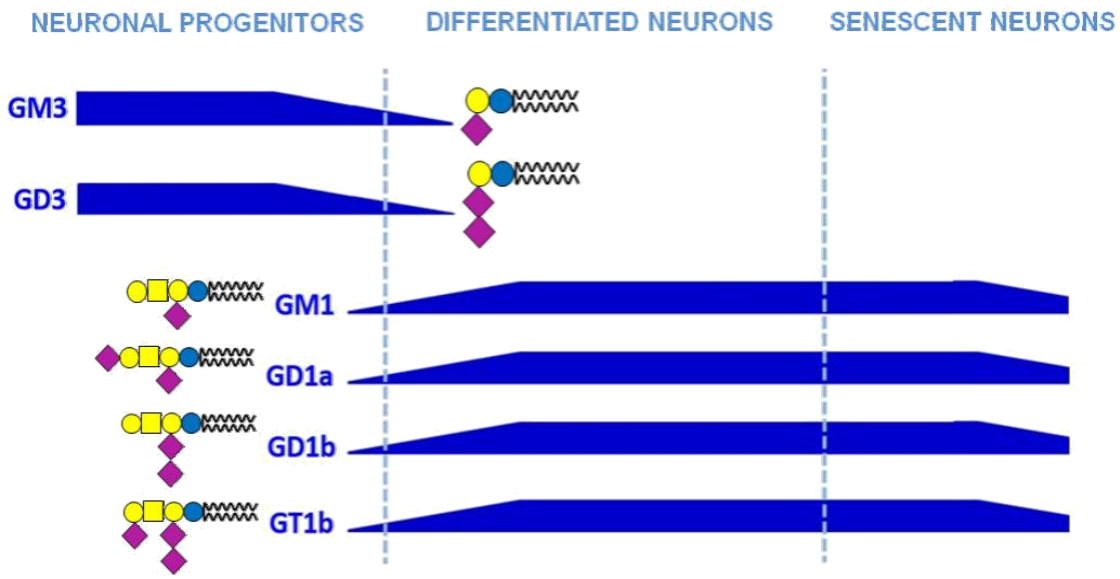


Figure I3: Ganglioside changing in neurodifferentiation. Modification in neuron ganglioside pattern during neurodifferentiation reflects the differences in glycosyltransferases and suggests an important role of oligosaccharide chains in the process (modified from Yu *et al*. 2008). Symbols for

ganglioside saccharide unites are in accordance with the glycan symbol nomenclature updated in the Second Edition of Essentials of Glycobiology (Varky *et al*. 2009).

This event is accompanied by a change in the expression of genes coding for the glycosyltransferases, and mainly the STII (GD3-synthase) which undergoes a downregulation during the brain maturation and the GalNacT (GM2/GD2 synthase) which is progressively upregulated (Yu *et al*. 2008).

The same trend is reproducible in primary cultures of cerebellar neuronal precursors obtained from rodents, which undergo a complete maturation process *in vitro* as already reported (Prinetti *et al*. 2001; Aureli *et al*. 2011).

The morphological change is accompanied by modifications in sphingolipids, particularly gangliosides, which content increased over other lipids (van Echten and Sandhoff 1988; Aureli *et al*. 2011). Thus, the surface density of gangliosides in the membrane of fully differentiated neurons is much higher than in undifferentiated cells. Moreover, the main ganglioside in precursor neuron is GD3, whereas GM1, GD1a and more complex gangliosides increase during differentiation and maturation with GD1a becoming the most represented ganglioside in mature neurons (Figure I4). During *in vitro* aging of primary cerebellar neurons GM1 undergoes impressive reduction resembling what occurs in the aging of the entire brain.

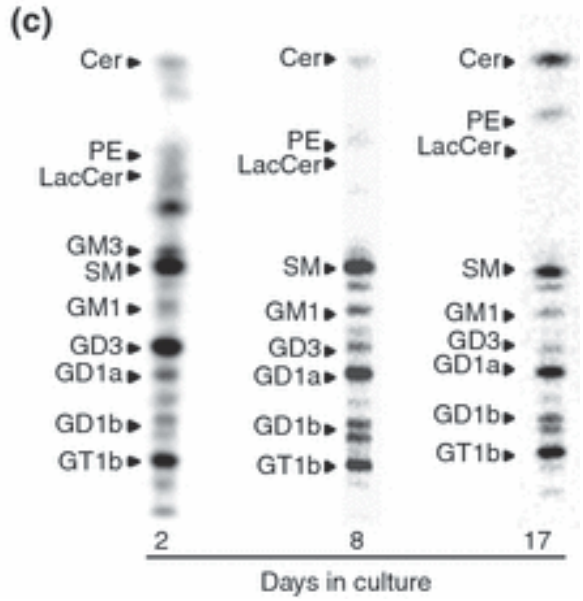


Figure I4. Changes in lipid patterns in rat cerebellar granule cells during development and aging *in vitro*. Patterns of radioactive lipids in homogenates obtained from rat cerebellar granule cells at the 2nd, 8th and 17th day in culture. Total content of ganglioside increases during

development. GD3 represents the most component in immature neurons, whereas during maturation complex gangliosides augment (modified from Aureli *et al*. 2011).

The importance of GM1 in modulating differentiation and neuritogenesis can be seen in numerous very earlier studies using neuroblastoma cells and primary neurons where the application of GM1 was shown to be directly associated with neuritic outgrowth (Ledeen 1984; Facci *et al*. 1984; Skaper *et al*. 1985; Ledeen *et al*. 1998). Mouse neuroblastoma cells N2a were one of the first and then widely neuron like model used to examine GM1 neuritogenic properties. The administration of exogenous GM1 to these cells was shown to induce neurite sprouting with a mechanism proposed to involve the cell plasma membrane (Facci *et al*. 1984). GM1 was also administered to primary culture of chicken and rat embryonic neurons deriving from both central and peripheric nervous system (Skaper *et al*. 1985), where it was found to accelerate neurite outgrowth under selected culture conditions. In the N2a neuroblastoma cells the same effect was obtained by the application of sialidase (Hasegawa *et al*. 2000) and was inhibited by the concomitant addition of Ctx-B (Wu *et al*. 1996). On the contrary, in the N18 neuroblastoma cells the concomitant addition of the toxin was necessary to observe neuritogenesis (Masco *et al*. 1991; Fang *et al*. 2000). Applied sialidase had similar axonogenic effects on spinal neurons (Yang *et al*. 2006; Mountney *et al*. 2010) even though in these studies the effect was ascribed to concomitant removal of polysialogangliosides associated with axon regenerators inhibitors. Da Silva and co-workers (Da Silva *et al*. 2005) demonstrated that in hippocampal neurons, the local enrichment of membrane sialidase, by its overexpression, was associated with the locally increase of GM1 and axon specification, a process dependent on a significant modulation of nerve growth factor (NGF) signal.

In PC12 pheochromocytoma cells, GM1 enhanced NGF-induced differentiation (Ferrari *et al*. 1995) and the latter was found to be subordinated to the presence of membrane GM1 as indicated by the abolished NGF responsiveness of PC12 cells following GM1 depletion (Mutoh *et al*. 1998).

3.2. GM1 in neuronal protection and repair

GM1 ability to protect cells from injuries and insults has been largely examined.

In N2a neuroblastoma cells the exogenous administration of GM1 protected cells from death caused by calcium ionophore application, suggesting the GM1 influence in modulating

excitotoxic effects due to massive calcium intracellular elevation. Similar effects have been observed in cultures of primary neurons exposed to glutamate. Glutamate represents the brain's main neurotransmitters which, through the binding to specific receptors, regulates neural communication, memory formation and learning. When neurons are exposed to massive glutamate concentration or for prolonged time periods, they are damaged or killed due to the excessive levels of calcium ions (Ca^{2+}) entering the cell, a process named excitotoxicity.

Glutamate cytotoxicity was reduced by GM1 in murine cerebellar granule cells (Bachis *et al*. 2002; Sokolova *et al*. 2014), and in cortical neurons (Park *et al*. 2016). In the latter study, electron spry mass spectrometry detected elevation of endogenous GM1 levels following the application of the neurotoxic stimulus to indicate a possible intrinsic defense mechanism against neuronal stress. In fact, neurons lacking complex gangliosides due to the ablation of *β4galnt1* gene (GM2/GD2 synthase) are more susceptible to elevated KCl and glutamate excitotoxicity and the application of GM1 rescued their phenotype (Wu *et al*. 2004).

GM1 antiperoxidative properties have been also exploited: in PC12 pheochromocytoma cells exposed to cytotoxic concentration of H_2O_2 (Zakharova *et al*. 2014) and in MPTP-susceptible cell lines (Saulino *et al*. 1993; Verma and Schneider 2019) ROS production leading to cells death, was prevented by exogenous GM1 application.

Induction of axonal damage to neurons of the central and peripheric nervous system followed by GM1 elevation allowed to examine the surprising reparative and axonogenic abilities of GM1. The axonal lesion in primary neurons of the peripheral nervous system leads to the local increase of GM1 due to the action of the Neu3 sialidase which starts the regeneration process (Kappagantula *et al*. 2014). This process is essential to induce axonal regrowth. Failure to activate sialidase in CNS neurons explains why these neurons are intrinsically unable to regenerate, a deficit found to be rescued by the induced sialidase overexpression (Rodriguez *et al*. 2001).

Many reports have examined neuroprotective and neurorestorative properties of GM1 in animal models as well as in clinical trials in the contexts of neurological and neurodegenerative pathologies.

Stroke-induced neurological damage is the field where the greatest clinical experience exists (Magistretti *et al*. 2019; Zhang *et al*. 2019). In several animal model and clinical trials,

systemic administration of GM1 was significantly more effective than placebo in improving neurological outcomes following a stroke.

The alteration of the ganglioside organization of the plasma membrane and of sphingolipid metabolism has been reported in neurodegenerative diseases and dementias (Piccinini *et al.* 2010; Breiden and Sandhoff 2018; Magistretti *et al*. 2019). This is particularly true for progressive neurodegenerative Parkinson's disease (PD), where the continuous death of nigro-striatal dopaminergic neurons leads to the onset of the characteristic motor and cognitive symptoms of the disease (Zesiewicz 2019, Del Tredici *et al*. 2002). In this context subnormal levels of GM1 have been associated with PD onset both in mouse and in humans (Forsayeth and Hadaczek, 2018; Wu *et al*. 2012). This is due to the recent evidence of lower levels of GM1 and expression of ganglioside synthesis genes in the neurons of the *substantia nigra* and occipital cortex of PD patients compared to healthy subjects of the same age (Schneider *et al.* 2018; Hadackzec *et al*. 2015). Furthermore, the total or partial ablation of GM1 ganglioside due to *B4galnt1* disruption in genetically modified mice, leads to the spontaneous development of the motor impairment and neuropathological lesions characteristic of human PD (Wu *el al*. 2011; Hadackzec *et al*. 2015).

Overall, in preclinical models recapitulating the characteristics of PD *in vitro* and *in vivo*, comprising non-human primates' PD models (Herrero *et al*. 1993; Rothbla 1998; Pope-Coleman 1998), strategies for increasing neuronal GM1 levels have demonstrated their effectiveness in preventing stress and nigrostriatal neurons' death (Schneider *et al*. 2019, 2015a; Xu *et al*. 2014, Hadjiconstantinou *et al*. 1989). Furthermore, GM1 demonstrated a modest but significant efficacy in clinical trials, where PD patients recorded improvements in motor and cognitive outcomes compared to the placebo-receiving counterpart (Schneider *et al*. 2010, 2013, 2015b).

The plethora of neurotrophic and neuroprotective effects of GM1 is dependent on the ability of the ganglioside to regulate multiple aspects of neuronal development and survival. Although the mechanisms underlying these effects are still awaiting to be clearly defined, two main molecular aspects are known so far to be strictly regulated by GM1: intracellular calcium levels and the neurotrophins-modulated pathways.

4. GM1 modulation of Calcium flux

It is known that calcium represents an important second messenger and its intracellular levels are finely regulated to orchestrate the processes of neuronal differentiation and survival. The identification of GM1 as a robust calcium flow modulator helped to define the molecular basis of the GM1 spectrum of action (Ledeen and Wu 2018).

4.1. GM1 influence on calcium influx

Elevation of GM1 on the surface of N2a and certain other neuroblastoma cells by exogenous sialidase induced calcium influx via channel identified as low-threshold voltage dependent T-type (Wu and Ledeen 1991; Fang *et al*. 2000). A similar phenomenon was observed in primary cultures of hippocampal neurons, where GM1-mediated axonogenesis was accompanied by local calcium influx (Rodriguez *et al*. 2001).

The regeneration of injured peripheral nerve promoted by GM1 elevation due to local Neu3-sialidase action in the membrane, is associated to the increase in calcium influx (Kappagantula *et al*. 2014). Such activation failed to occur in injured retinal neurons of central nervous system despite the presence of Neu3 in the tissue. This was suggested to account for the failure of injured central nervous system axons to regenerate in contrast to the ability of peripheric nervous system axons to do so. Also the use of the Ctx-B as GM1-crossilinking agent was shown to induce calcium influx in both central (Wu *et al*. 1996) and peripheric neurons (Milani *et al*. 1992).

An alternative mechanism of intracellular calcium regulation by GM1 involves the transient receptor potential channel 5 (TRPC5), observed by the employment of crosslinking agents on primary central and peripheric neurons. In these studies, a co-crosslinking with proteins associated with GM1 occurred, such as integrin heterodimer α5β1, which in turn activated an adhesion kinase and the downstream pathway leading to the opening of the TRPC5 channel (Wang *et al*. 2009).

4.2 GM1 influence on calcium efflux

The finding that GM1 protected neurons from the toxic effects induced by glutamate and the calcium ionophore (paragraph above) suggested a regulation of intracellular calcium homeostasis including also its extrusion.

Calcium efflux from the neuronal cytoplasm was found to be under GM1 regulation at least via two mechanisms: plasma membrane calcium ATPase (PMCA) and Na^+/Ca^{2+} exchanger (NCX). PMCA accounts for the high affinity low capacity system representing the major system regulating calcium efflux while NCX is the opposite. In neurons membrane PMCA isoform 2 is the most expressed (Burette *et al*. 2003) and was found to be slightly inhibited by GM1 (Zhao *et al*. 2004). On the other hand, NCX was found to be associated with and regulated by GM1 (Xie *et al*. 2002). The presence of the NCX associated with GM1 in the nuclear envelope has suggested a mechanism of regulation of nucleoplasmic calcium levels. The NCX/GM1 complex mediates the transfer of calcium from the nucleoplasm to the luminal space of the nuclear envelope, which is continuous with the lumen of the endoplasmic reticulum, serving as a protection mechanism for the nucleus against the action of enzymes activated by the increase in calcium levels (Wu *et al*. 2009).

A schematic summary of the regulation of calcium flows by GM1 at various cellular levels is shown in the Figure I5.

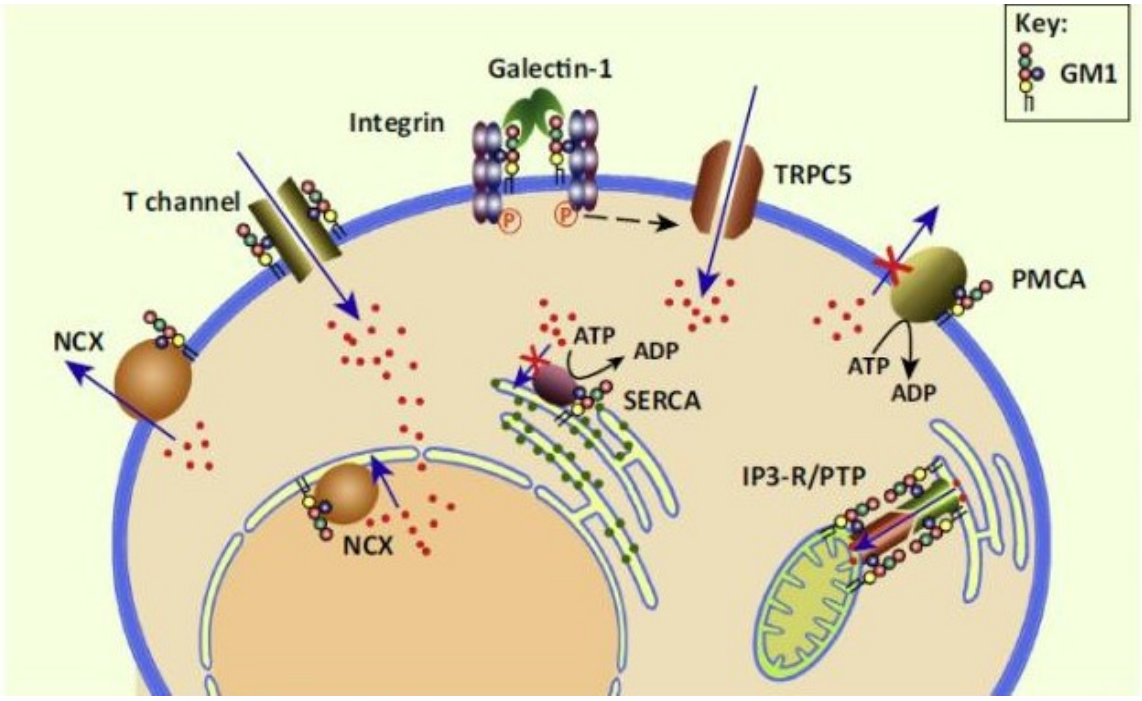


Figure I5: Summery of GM1 influence on regulation of calcium flux. GM1 can interact directly or indirectly with different proteins responsible to mediate influx or efflux of Ca^{2+} across cell membranes. NCX, Na^+/Ca^{2+} exchanger; TRPC5, transient receptor potential channel 5; PMCA, plasma membrane Ca^{2+}-ATPase pump; SERCA, sarco/endoplasmic Ca^{2+}-ATPase pump; PTP, IP_3-R, inositol-3-phosphate receptor; PTP, permeability transition pore (Ledeen and Wu 2015).

5. GM1 modulation of neurotrophic signal

5.1. Neurotrophins and neurotrophic factors receptors

Neurotrophic factors, such as the family of neurotrophins, are recognized for their role in neuronal differentiation, survival and maturation. The long-term survival of neurons is due to continuous support from neurotrophins, and the progressive decrease in support derived from the underlying signaling contributes to neurodegeneration. The neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, NT-4/5 and NT-6. These factors act by binding to transmembrane tyrosine kinase receptors (Trk) and p75 neurotrophin receptor (p75NTR), promoting receptor activation (Huang, 2001). In particular, NGF binds to TrkA; BDNF and NT 4/5 bind to TrkB; NT-3 primarily activates TrkC but can also bind TrkA and B. p75NTR binds to all neurotrophins with a lower affinity then the others Trk. Neurotrophins' binding to Trk induces receptor tyrosine kinase dimerization and autophosphorylation, therefore initiating a complex cascade of signal transduction events. Tyrosine phosphorylated Trk binds to Shc, phospholipase C and phosphatidyl inositol 3-kinase (PI3K) through SH2 domains resulting in their phosphorylation and subsequent activation of intracellular signaling pathways and transcription factors regulating gene expression (Kaplan and Stephens 1994; Klesse and Parada 1999).These pathways transduce signals independently, but also can converge on the same downstream effector. Stimulation of a variety of tyrosine kinase receptors leads to a rapid elevation of the enzymatic activity of a family of structurally related serine–threonine kinases, known as mitogen-activated protein kinases (MAPKs), which convert extracellular stimuli to intracellular signals that control gene expression (Schaeffer and Weber 1999).

Members of other neurotrophic factor families have also been shown to activate tyrosine kinases. These include glial-cell-line-derived neurotrophic factor (GDNF) family ligands (GFLs). GFLs bind to specific GDNF family receptor α (GFRα) proteins, all of which form receptor complexes and signal through the RET receptor tyrosine kinase. Among all components of the family, GDNF has been largely studied for its importance in regulating the survival of dopaminergic neurons (Sariola *et al*. 2003). GDNF displays highest affinity for GFRα1. The receptor complex GDNF–GFRα1 binds to the extracellular domain of RET receptor modulating various intracellular signaling cascades, comprising PLC, PI3K and MAPK pathways sustaining differentiation, maturation and survival of neurons.

A summary of the intracellular pathways activated by neurotrophic receptors is outlined in Figure I6

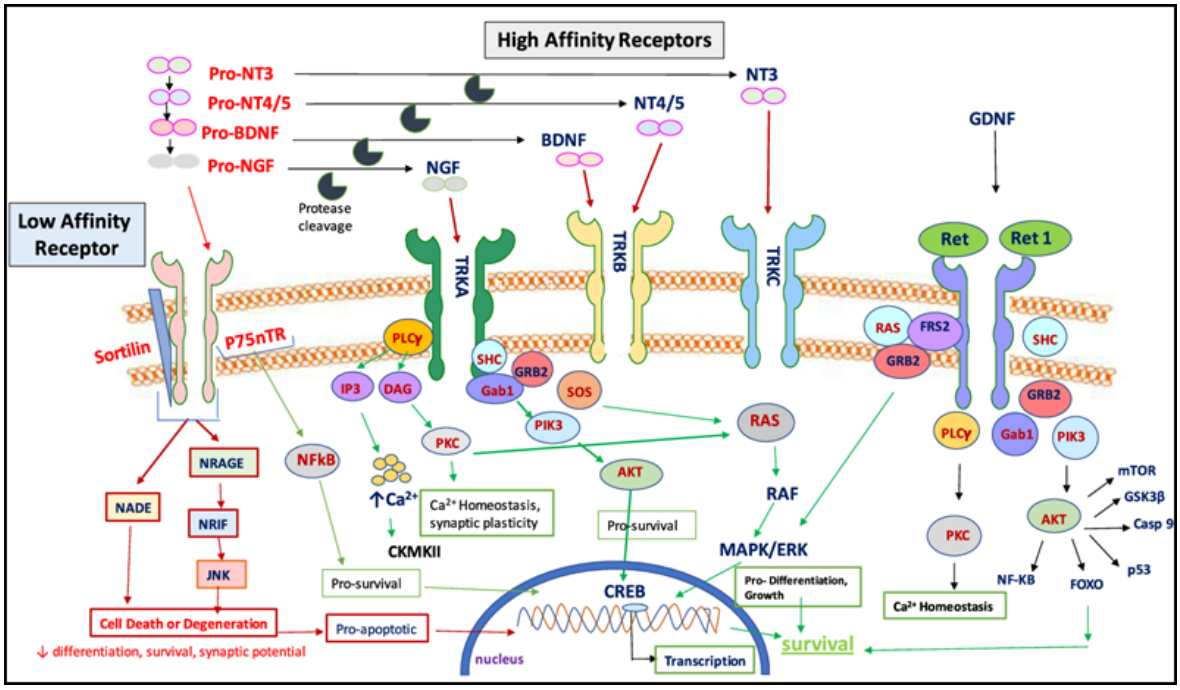


Figure I6. Schematic illustration of neurotrophins' signaling sustaining neuron differentiation, growth and survival. NT = neurotrophins, BDNF = brain-derived neurotrophic factor, TRK = tyrosine receptor kinase, GDNF = glial-derived neurotrophic factor, PLC = phospholipase C, RAS = Ras proteins, GRB = growth factor receptor-bound protein 2, PIK3 = phosphatidylinositol-4,5-bisphosphate 3-kinase, AKT =AKT serine/threonine–protein kinases, FOXO = The forkhead box O transcription factor, PKC = protein kinase C, CREB= the cAMP-responsive element-binding protein (modified from Kashyap *et al*. 2018).

5.2. GM1 influence on neurotrophic receptors

Neurotrophins and their receptors have been shown to be GM1 substrates, contributing to explain the neurotrophic and protective effects of the ganglioside (Ferrari *et al*. 1995; Mutoh *et al*. 1995; Farooqui *et al*. 1997; Bachis *et al*. 2002). Earlier studies demonstrated that GM1 was able to activate TrkA receptor in different cell lines (Mutoh *et al*. 1995; Rabin *et al*. 1995) and primary neurons (Da Silva *et al*. 2005), suggesting GM1 may mimic NGF activity in inducing neuronal differentiation and neuritogenesis. Moreover, in injured peripheric neurons the local increase of GM1 level due to the activation of Neu3 sialidase is accompanied by ERK1/2 activation which drives axonogenesis. (Kappagantula *et al*. 2014). The application of GM1 to *ex-vivo* cultures of striatum, frontal cortex and hippocampus of adult rats resulted in the activation of Trks and ERKs, and the effect was confirmed by *in vivo* intraventricular injection of the ganglioside (Duchemin *et al.* 2002).

Also the protective role of GM1 has been found depending on TrkA activation (Sokolova *et al.* 2014) coupled to ERK1/2 and Akt downstream pathways (Zakharova *et al*. 2014).

In addition to what found about TrkA receptor, TrkB appeared to be modulated by the presence of GM1 in the plasma membrane of cultured neurons (Pitto *et al*. 1998; Bachis *et al*. 2002).

A similar finding was reported for the GDNF receptor. GM1 was shown to associate with the GDNF receptor complex comprised of Ret, the tyrosine kinase component, and GFRα, a GPI-anchored coreceptor; Ret association with GFRα was severely impaired in neurons totally or even partially devoid of GM1 (Hadaczek *et al*. 2015). Also the downstream MAPK phosphorylation was found lower in GM1 deficient neurons, which appeared to be restored by the administration of a GM1-analogue.

6. GM1-oligosaccharide as the bioactive portion of GM1

Despite the many evidence about the neurotrophic and neuroprotective properties of GM1, both from a physiological and pharmacological point of view and although some molecular partners of GM1 have been identified, the mechanism of action underlying its properties remained elusive.

Significant studies were carried out using GM1 derivatives keeping the same oligosaccharide structure, with the replacement of the long chain fatty acid of ceramide by a shorter group, such as acetylene (LIGA 4) (Manev *et al*. 1990) or dichloroacetylene (LIGA20) (Costa *et al*. 1993; Kharlamov *et al*. 1993; Saito *et al*. 1999; Rabin *et al*. 2002), represented in Figure. I7.

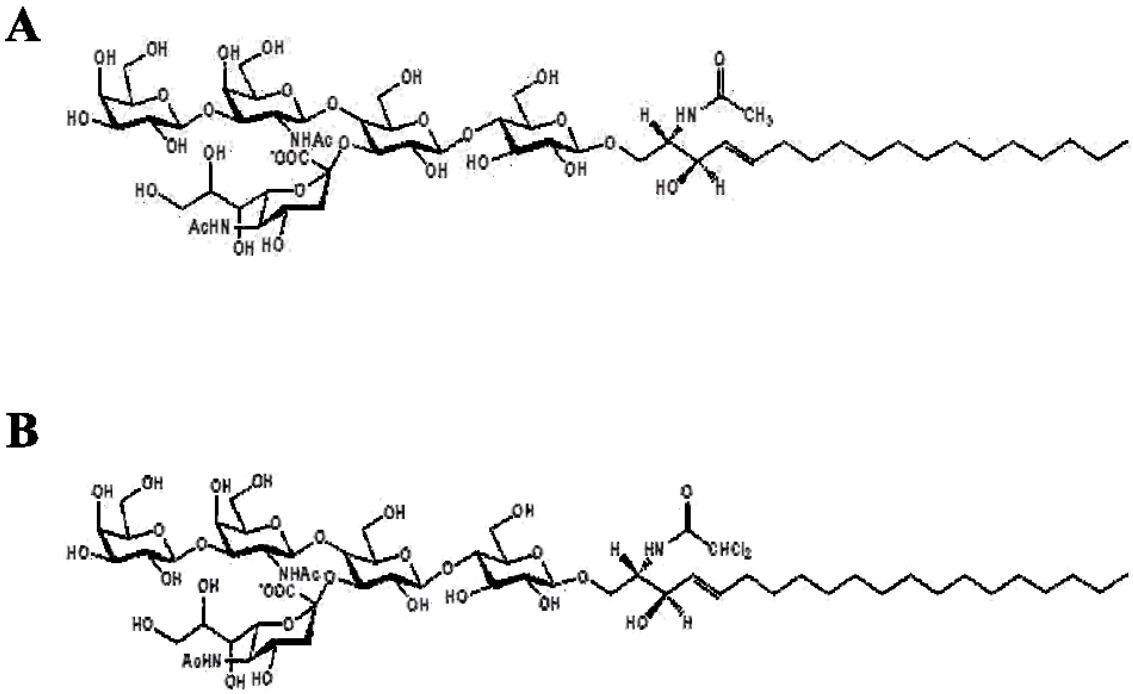


Figure I7. Semisynthetic GM1 derivatives chemical structures. A: LIGA 4; B: LIGA 20

In these studies, GM1 derivatives have proved to exert similar effects to those of GM1 with the involvement of Trks and RET receptors. Their remarkable difference from the parent compound resides in the incremented membrane permeability and, therefore, enhanced capability to penetrate the blood–brain barrier (BBB) and neuronal plasma membrane. Nevertheless, it was evident that the structure of the ceramide did not seem indispensable for GM1 to exercise its properties.

Furthermore, the above mentioned evidence of neurodifferentiating and protective effects observed deriving from the manipulation of endogenous GM1 following the modulation of the activity of membrane sialidase Neur3, suggests an important influence of the oligosaccharide chain in the fine-tuning of the processes beginning at the level of plasma membranes.

However, the pioneering work of Schengrund and collaborators (Shengrund and Proudy 1988) showed much earlier that the GM1 oligosaccharide (oligoGM1) alone maintained the neuritogenic properties of GM1 in neuroblastoma cells S20Y.

More recent studies have investigated the oligoGM1 differentiative properties, confirming its neuritogenic potential in N2a murine neuroblastoma cells (Chiricozzi *et al*. 2017). In this

study the addition of oligoGM1 in the culture medium induced the emission of neuritic prolongations and the elevation of the Neurofilament proteins analogously to what was observed administering the entire GM1 as shown in Figure I8.

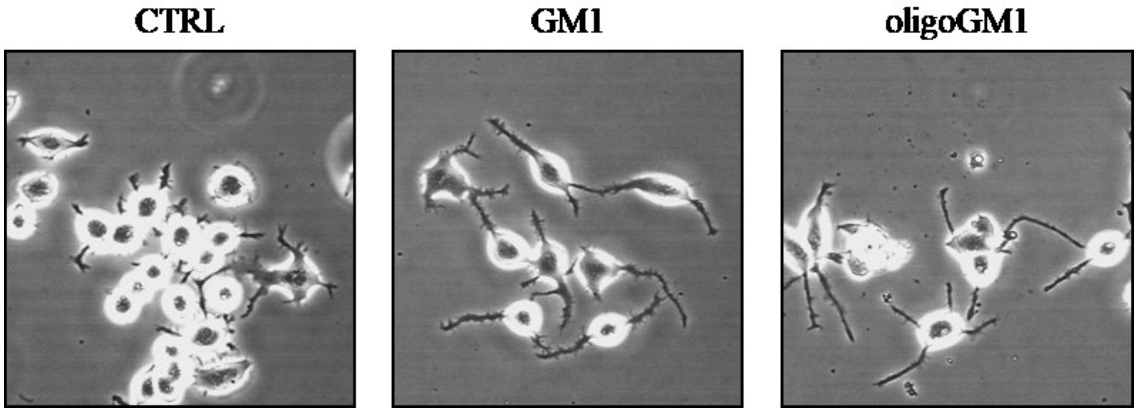


Figure I8. Neurodifferentiation of N2a cells induced by GM1 and oligoGM1. OligoGM1 induced neuritogenesis is comparable to the one induced by GM1 on N2a neuroblastoma cells (Chiricozzi *et al*. 2017).

Incubation with sugar derivatives, Gg_4, $II^3Neu5Ac\text{-}Gg_3$, sialyllactose, galactose, or sialic acid was performed in order to clarify the minimal structure required to promote neurite elongation. None of these compounds was found able to induce cell morphological changes. Interestingly, the administration of a GM1-oligosaccharide derivative containing a α-fucose in the second position of the external galactose, was shown to induce neurite sprouting and reduction of cell proliferation in a similar way as $II^3Neu5Ac\text{-}Gg_4$. This result suggested that the β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-Glc represented the minimal structure required by the saccharide to exert its neuronal function and the addition of an α-fucose at position 2 of the external galactose was irrelevant for the process.

In time-course experiments employing a radiolabeled form of oligoGM1, the researchers found no trace of radioactivity inside the cells, concluding that the morphological effect depended on an action at the level of the cellular surface (Chiricozzi *et al*. 2017).

Investigating the molecular pathway promoted by oligoGM1 in more detail, the researchers found that the sugar induced an increase in TrkA receptor phosphorylation on tyrosine 490, accompanied by an increase in MAPK ERK1/2 activation. GM1 induced the same pathway at a comparable extent and a specific TrkA inhibitor abolished oligoGM1 induced neuritogenesis (Scheme in Figure I9)

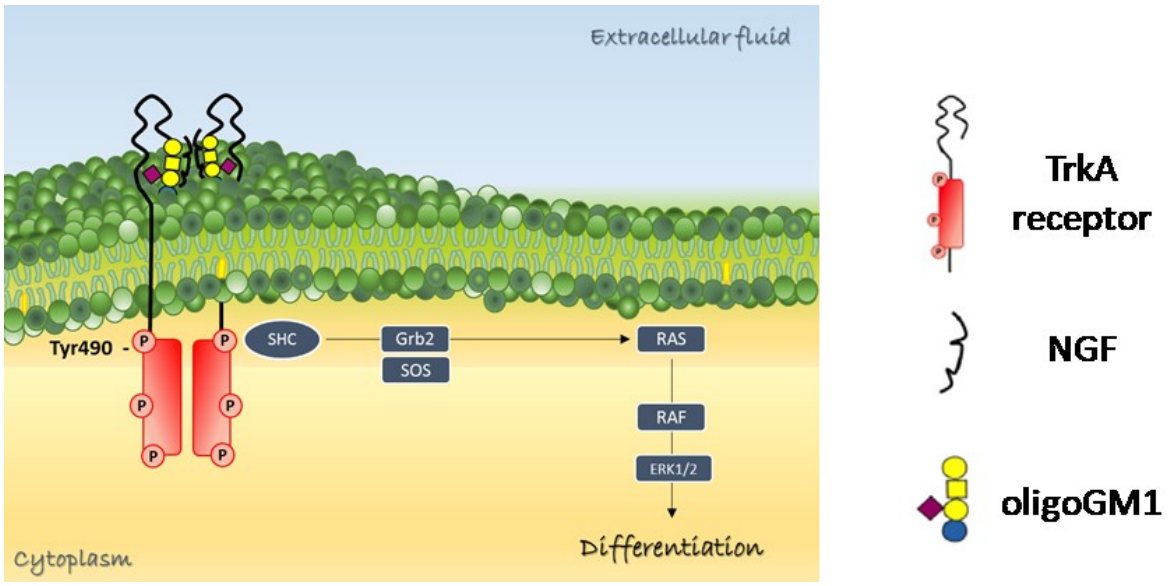


Figure I9. Scheme of the proposed mechanism underling GM1 neuritogenic effect in N2a. GM1 enhances TrkA phosphorylation on tyr490, throughout its oligosaccharide component. TrkA activation induces MAPK differentiative pathway. ERK, extracellular signal-regulated protein kinases 1 and 2; Grb2, growth factor receptor-bound protein 2; Gab1, Grb2-associated binder-1; RAS, GTP-binding protein; RAF, serine/ threonine kinase; SHC, transforming protein 1; SOS, son of sevenless (Chiricozzi *et al*. 2017).

By photolabeling experiments performed with a nitrophenylazide containing oligoGM1, labeled with tritium, a direct interaction between the GM1 oligosaccharide and the extracellular domain of TrkA receptor was shown (Figure I10).

This discovery demonstrated for the first time a direct association between the NGF receptor and one specific portion of GM1, i.e. the oligosaccharide.

This evidence was confirmed in the subsequent study (Figure I10) that employed two modified forms of radiolabeled GM1: one carrying a photo-activatable phenylazide group associated with the oligosaccharide chain, and the other one carrying the same group within the lipid portion (Chiricozzi *et al*. 2019a). After administration of these two derivatives to the N2a, the cells were irradiated with UV light. The proteins were then extracted, separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was subjected to autoradiography and subsequently incubated with TrkA specific antibody. The overlapping between the radioactive and the chemiluminescent signals was obtained only by using the radiolabeled-GM1 derivative containing the photoactivable group inserted in the oligosaccharide head. This finding clearly indicated that the interaction between TrkA and ganglioside GM1 occurred outside the cell at the level of the oligosaccharide.

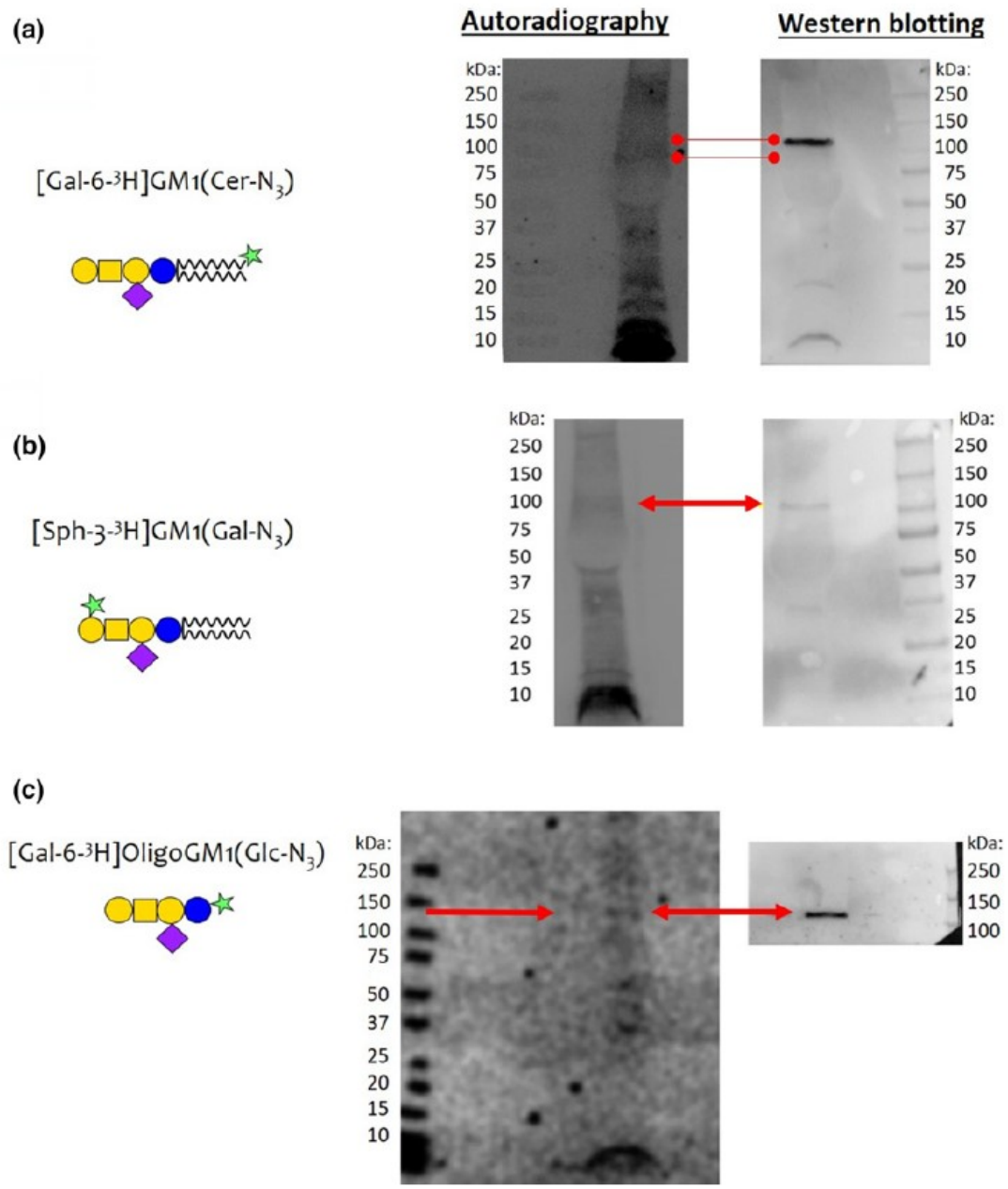


Figure I10. Interaction between TrkA and GM1 occurs at the level of GM1-oligosaccharide in N2a cells. Radiolabel derivative of GM1 carrying the photoactivable group, represented by the green star in the symbolic representation, were administered to cells. UV light was used to induce crosslinking between GM1-derivates and surrounding molecules. After radiography of cell protein pattern and TrkA immunoblotting, overlapping between radio-signal and chemiluminescent signal was only found with oligoGM1 and GM1 carrying photoactive group on the oligosaccharide (Chiricozzi *et al*. 2017, 2019a)

Accordingly, molecular docking analyses confirmed that GM1 oligosaccharide binds the TrkA-NGF complex significantly reducing the free energy of association, acting as a bridge able to increase and stabilize the TrkA-NGF molecular interactions (Figure I11)

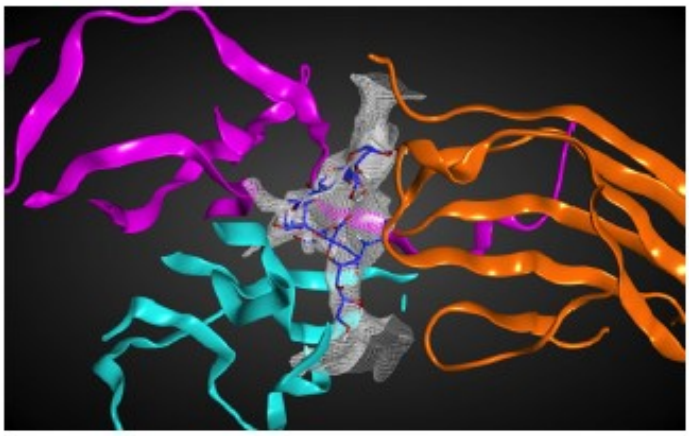


Figure I11. Modeling the interaction between NGF-TrkA complex and oligoGM1. TrkA in orange ribbons; two NGF molecules: one in cyan ribbons and one in magenta ribbons. OligoGM1 is represented in sticks, with blue color for carbon atoms and red color for oxygen atoms. Van der Waals interaction surface between oligoGM1and proteins is represented as a white mesh map (Chiricozzi *et al*. 2017).

These studies are beginning to shed new light on the mechanism of action of the GM1 neurotrophic effects, identifying in its oligosaccharide component the portion responsible for its neuritogenic properties, at least in neuroblastoma cells.

Therefore, in the presence of physio-pathological alterations of the membrane ganglioside composition, it is becoming important to focus attention on the contribution given by the change in the oligosaccharide composition. In this perspective, the increase in GM1 levels, for instance, locally enriches the glycocalyx of oligoGM1 molecules which are likely responsible for downstream biosignaling.

The question remains whether in primary neurons, the oligosaccharide is able to exert a similar effect, going to replace the role of the whole ganglioside.

Aim

The neurotrophic properties of GM1 have been extensively studied both *in vitro* and *in vivo*. Regardless of the approach used, the conclusions of all the studies led to the assertion that the increase in GM1 content at the plasma membrane level favors neuronal differentiation, maturation and survival. Despite this certainty, the molecular basis of the mechanism of action of GM1 is currently unclear. Even the portion indispensable of the biological role of GM1 was obscure until two years ago, when the discovery that GM1 oligosaccharide corresponds to the bioactive portion of GM1, which alone exerts the same differentiating effect as the entire GM1 in N2a neuroblastoma cells, allowed to shed new light in this field (Chiricozzi *et al*. 2017).

Often the bio-active properties of amphiphilic lipids such as gangliosides have been attributed to the hydrophobic component which, through alteration of membrane physical properties, or through the direct interaction with membrane proteins, could influence intracellular signaling (Chiricozzi *et al*. 2015; Loberto *et al*. 2003). Aside of the ceramide, the hydrophilic head of glycolipids, with a specific order and composition of saccharide units, can regulate the functioning and activation of membrane proteins and therefore modulate entire signal transduction pathways. In the case of GM1, its oligosaccharide portion was found to interact directly with the extracellular portion of TrkA, the specific receptor for NGF, stabilizing the complex between the ligand and the receptor and therefore favoring TrkA activation. The increase in TrkA phosphorylation was associated with the elevation of the MAP kinases activity responsible for blocking N2a proliferation and induction of differentiation.

The raising question was whether GM1 oligosaccharide could influence neuronal differentiation and development in a more physiological context. The aim of the doctoral project was to evaluate the effects of oligoGM1 in neuronal progenitors both from a morphological and a molecular point of view, trying to define the mechanism by which GM1 molecule exercises its neurotrophic role.

For this purpose, primary cultures of cerebellar granular neurons were used. These cells differentiate and mature *in vitro* allowing the study of physiopatological processes such as neuronal development, maturation and aging. Furthermore, these cells have been widely used for the study of the lipid pattern and its dynamics during neuronal aging. More importantly, it has been shown that in granular cells, GM1 is able to influence the activity of Trk receptors (Rabin *et al*. 2002). In the present study, the oligosaccharide chain of GM1 was employed with the purpose to provide knowledge regarding the mechanism by which

the specific sugar code of GM1 influences neuronal differentiation through the modulation of plasma membrane signaling.

Materials & 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.

Phosphate buffered saline (PBS), paraformaldehyde (PFA), RNAase-free water, sodium orthovanadate (Na_3VO_4), phenylmethanesulfonyl fluoride (PMSF), aprotinin, and protease inhibitor cocktail (IP), (Ctx-B-HPR), o-phenylenediamine tablets, H_2O_2, bovine serum albumin (BSA), donkey serum and rabbit polyclonal anti-GADPH (RRID:AB_796208) antibody were from Sigma-Aldrich (St. Louis, MO, USA). Fetal Bovine Serum (FBS), L-Glutamine and penicillin/streptomycin (10.000 Units/ml) were from EuroClone (Paignton, UK). Rabbit anti-TrkA (RRID: AB_10695253), rabbit anti–extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (RRID: AB_390779), rabbit anti-phospho-ERK (p-ERK1/2, Thr202/Tyr204) (RRID: AB_2315112), goat anti-rabbit IgG (RRID: AB_2099233), mouse anti-β3-tubulin (RRID:AB_1904176), rabbit anti-phospho-Src (RRID:AB_331697), rabbit anti-Synapsin (AB_2200102) antibodies were from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-phospho-TrkA (Tyr490) (RRID:AB_628399) antibody was from Santa Cruz Biotechnology (Heidelberg, Germany). Mouse anti-neuroglycan C (RRID not available), mouse anti-phospho-FAK (Tyr 397) (RRID:AB_399286), mouse anti-FAK (RRID:AB_397494) was from Becton Dickinson. Chemiluminescent kit for western blot was from Cyanagen (Bologna, Italy). Rabbit anti-pan Neurofilament (NF) antibody (RRID: AB_10539699) was from Biomol International (Plymouth Meeting, PA, USA). Neurobasal A medium, B27 Supplement, 4',5-Diamidina-2-phenylindole (DAPI), DNase I, SuperScript™ III First-Strand Synthesis System, Oligo$(dT)_{20}$ Primer and SYBER Green Master Mix were from Thermo Fischer Scientific (Waltham, MA, USA). Ultima gold was from Perkin Elmer (Waltham, MA, USA). 4 –20% Mini -PROTEAN® TGX™ Precast Protein 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) plate and Triton X -100 were from Merk Millipore (Frankfurten, Germany).

Methods

1. Chemical synthesis and preparation of gangliosides GM1 and GD1a and their oligosaccharides

1.1 Gangliosides' purification

GM1 and GD1a gangliosides were 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). High amount of GM1 was obtained by the sialidase treatment of the total pig brain ganglioside mixture. This simplified the purification process as the major part of polysialogangliosides were transformed into GM1 (Acquotti *et al*. 1990). 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. Incubation at 36°C and 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.

1.2 Radiolabeled GM1

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) Figure M1.

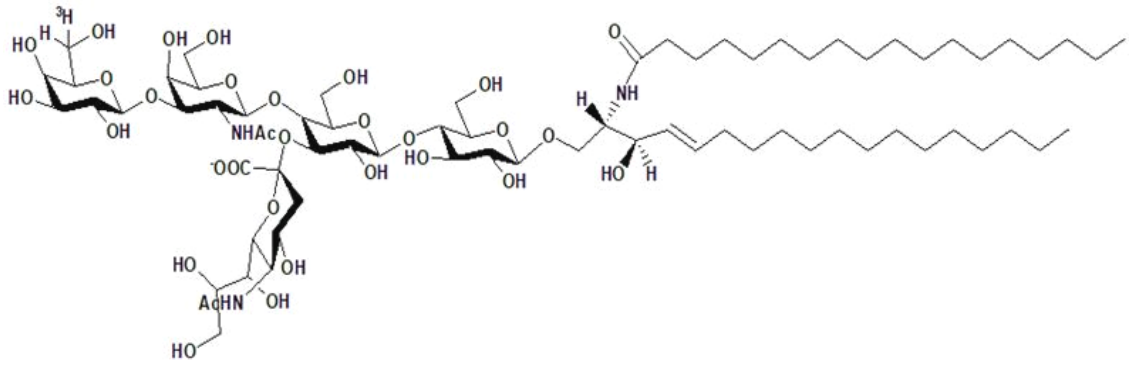


Figure M1: [Gal-6-^{3}H]GM1 structure.

1.3 Gangliosides' oligosaccharides

The oligosaccharides II^{3}Neu5Ac-Gg$_{4}$, II^{3}Neu5Ac-[^{3}H]Gg$_{4}$ and IV^{3}AcII^{3}Neu5Ac-Gg$_{4}$ were prepared by ozonolysis followed by alkaline degradation (Wiegandt and Bucking 1970), from GM1, [^{3}H]GM1 and GD1a respectively (Figure M2). Briefly, GM1, [^{3}H]GM1 or GD1a was dissolved in methanol and slowly saturated with ozone at 23°C. The solvent was then evaporated under vacuum and the residue brought immediately to pH 10.5-11.0 by addition of triethylamine. After solvent evaporation, GM1 and GD1a oligosaccharides were purified by flash chromatography using chloroform/methanol/2-propanol/water 60:35:5:5 v/v/v/v as eluent. Oligosaccharides were dissolved in methanol and stored at 4°C.

Altogether, NMR, MS, HPTLC and autoradiographic analyses showed a homogeneity over 99% for all the prepared gangliosides and oligosaccharides.

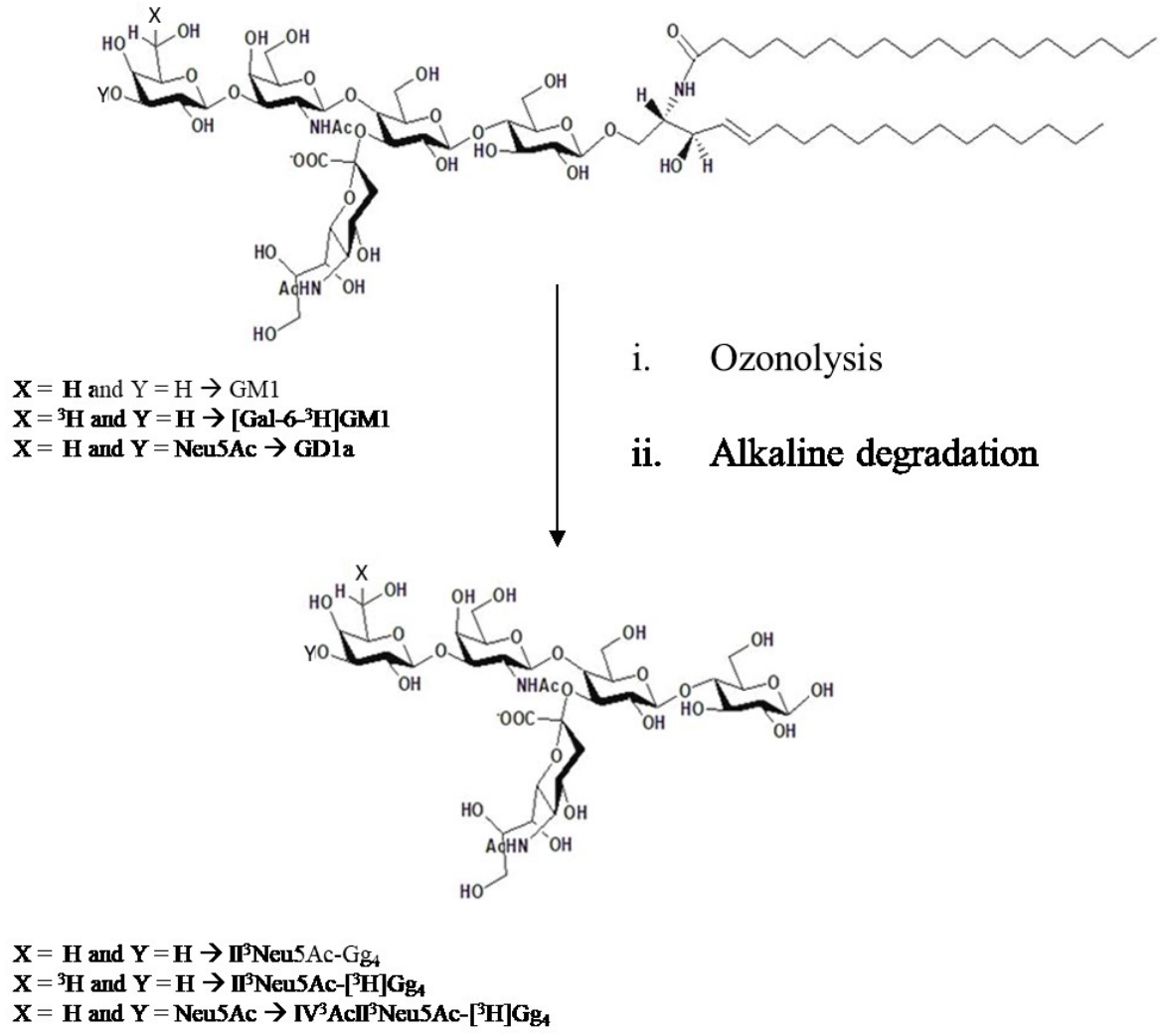


Figure M2: Chemical synthesis of oligoGM1 and [Gal-6-^{3}H]-oligoGM1 and oligoGD1a. Ozone-alkali fragmentation procedure was used to obtain desphingosino-gangliosides. Ozonolysis organic reaction cleaved unsaturated bond between sphingosine 4 and 5 carbons. Subsequently, the alkaline degradation with *triethylamine* released the oligosaccharide chain form the residue.

2. Primary culture of cerebellar granule neurons (CGN)

Pregnant C57BL/6J mice were provided by Charles River Laboratories International (project numbers 946/2017-PR and 255/2019-PR). All animal procedures were approved by the Ethics Committee of the University of Milano, Italy and were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (Directive 2010/63/EU). CGN were prepared as previously described (Bilimoria, 2008) with some modifications. Briefly CGN were dissociated from pooled cerebella of 5-day-old pups by mechanic trituration with blade (35 plus 35 times in perpendicular directions), followed by

incubation with 1% (W/V) Trypsin plus 0,1% (w/v) DNase I in PBS (1 mL every 5 cerebella) for 3.5 min at 23°C. The reaction was stopped by centrifugation of the cell mixture at 1000 *x g* for 10 sec and the supernatant was removed. The cell pellet was resuspended in the solution of 0.04% (w/v) Trypsin inhibitor plus 0.1% DNase I in PBS (1 mL every 5 cerebella). The cells were definitively dissociated by repeated passages through descending caliber glass Pasteur.

The reaction solution was removed by centrifugation and the cells were washed with 0.2% glucose in PBS before being resuspended in Neurobasal A medium containing 25 mM KCl, 1% B27 Supplement, 1% L-Glutamine and 1% penicillin/streptomycin. Cells were counted with a Burker chamber, plated at a density of 315000 cells/ cm^2 on plastic or glass coverslips precoated with poly-L-lysine (10 µg/mL for 2 h at 37°C) and maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2.

As shown in Figure M3, CGN maturation process consists in a first phase of cell aggregation followed by neurite elongation up to the formation of a neural network in three days and becoming adult neurons in 1 week.

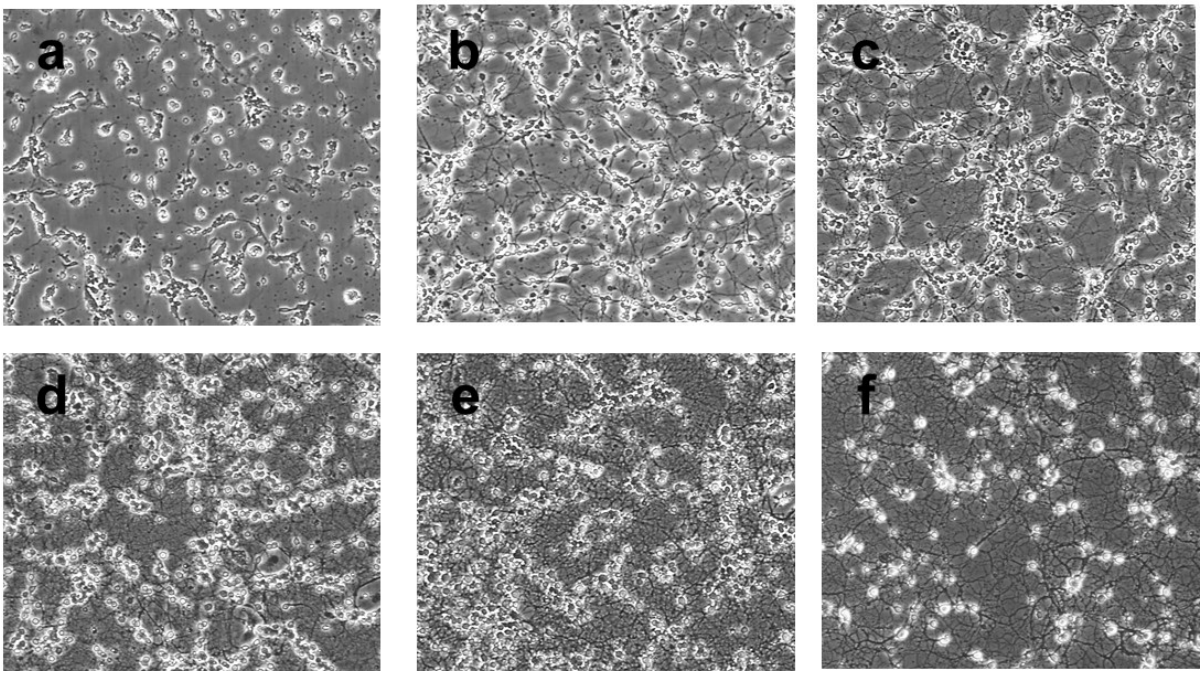


Figure M3. Differentiation and maturation of the CGN. Representative images of phase-contrast optical microscopy of CGNs dissociated from cerebellum tissue of P5 mice and grown *in vitro*. 100X magnification. a) 1 DIV; b) 2 DIV; c) 3 DIV; d) 7 DIV; e) 14 DIV; f) 20 DIV. DIV = Day *In Vitro*.

2.1 CGN treatment

Gangliosides and oligosaccharides were solubilized in methanol. To perform cell treatment, they were dried under nitrogen gas and dissolved in culture medium by vortex agitation and sonication in water bath 3 times for 30 sec. Solubilized gangliosides and saccharides were administered to cells during plating at the final concentration of 50 μM (Chiricozzi *et al*. 2017). Control cells were incubated under the same experimental condition but omitting any other addition.

3. Morphological analysis

CGN treated with gangliosides or oligosaccharides (50 μM) were observed over times by contrast phase microscopy with Olympus BX50 microscope (Olympus, Tokyo, Japan), with a 10X objective to final magnification of 100X. At least 10 random fields from each well were acquired from 8 different experiments.

Neuronal migration and motility were examined by live cell analysis. CGN seeded in Corning 6 well plates were immediately incubated in the IncuCyte® S3 system (Sartorius Stedim Biotech) to the automated live cell recording in phase contrast with 10X objective. 16 fields per well were randomly acquired every 2 h up to 24 h, in triplicate, in three different experiments. The analysis of the dynamics of neuronal clusters was performed automatically by the IncuCyte® S3 Software (V2018A, Sartorius Stedim Biotech), setting a segmentation index equal to 0.2. In this way the program identified objects related to clusters of neuronal cells in each frame, highlighting them in yellow. Finally, the count of the area occupied by the objects defined as clusters with respect to the area of each field returned the percentage of neuronal clusters' confluence for each frame.

4. Immunofluorescence analysis

CGN were seeded onto glass coverslip for 24 h or 48 h in the presence or in the absence of 50 μM oligoGM1. Then cells were fixed in 4% PFA in PBS for 20 min at 23°C. Cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min and then treated with a blocking solution containing 10% donkey serum in PBS for 1 h at 23°C. Cells were incubated with rabbit polyclonal antibody anti-Neurofilament (NF) for 2 h at 23°C. Cells were then rinsed in PBS and incubated 1 h with secondary anti-rabbit FITC-conjugated antibody. Nuclei were then detected by reaction with DAPI reagent 10 min at 23°C. Fluorescence microscopy was

carried out using an Olympus U-RFL-T EPI Fluorescence Microscope (Olympus, Tokyo, Japan) and the images' processing was performed with ImageJ software *(*ImageJ, NIH http://rsb.info.nih.gov/ij/). At least 10 fields for each condition were acquired.

5. Fate of oligoGM1 added to the cells

Fate of oligoGM1 administered to CGN was determined using tritium-labeled oligoGM1 on the external galactose saccharide (β-[6-^{3}H]Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-Glc). CGN were incubated in the presence of 50 μM [^{3}H]oligoGM1 for different times. Then the medium was removed and the following treatments were performed sequentially: (i) cells were washed three times with 10% FBS-medium to remove and collect the fraction of oligoGM1 weakly associated to the cells — serum removable fraction; (ii) cells were incubated with 0.1% trypsin solution for 1 min to remove the fraction of the oligoGM1 strongly linked to extracellular domain of plasma membrane proteins — trypsin removable fraction; (iii) cells were lysed in order to obtain the fraction of oligoGM1 internalized by the neurons — trypsin stable fraction. The radioactivity associated to each fraction was determined by liquid scintillation counting. The procedure was established previously to determine the fate of gangliosides and GM1 oligosaccharide administered to cells in culture (Chigorno *et al*. 1985; Chiricozzi *et al*. 2017).

6. Protein determination

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

7. Protein analysis

CGN grown in the presence or in the absence of oligoGM1 (50 μM) were washed twice with 1 mM Na_3VO_4 and lysed with 1 mM Na_3VO_4, 1 mM PMSF, 2% (v/v) aprotinin, and 1% (v/v) IP in cold PBS. Equal amounts of proteins were denatured, separated on 4-20% precast polyacrylamide gels, and transferred to PVDF membranes using the Trans -Blot® Turbo™ Transfer System (Bio -Rad). The presence of Neuroglycan C, β3-Tubulin, Synapsine I, FAK, P-FAK (tyr 397), Src, P-Src (tyr 416), TrkA, P-TrkA (tyr 490), ERK1/2 and P-ERK1/2 (Thr202/Tyr204) was determined by specific primary antibodies, followed by reaction with

secondary HPR-conjugated antibodies. GADPH was used as loading control. The data acquisition and analysis were performed using Alliance Uvitec (Eppendorf, Germany).

8. mRNA analysis

CGN grown in the presence or in the absence of oligoGM1 were lysed with TRIzol RNA Isolation Reagent (Thermofisher) followed by phase separation in chloroform and RNA isolation with isopropanol following manufacturer's instructions. RNA pellet was air dried and solubilized in 20 µL RNAase-free water. RNA yield was determined by The NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermofisher). Following reaction with DNAse I (2,73 U/µL) for 15 min at 23°C, 1 µg RNA was reverse transcribed by Superscript III First-Strand Synthesis System kit using Oligo-dT_{20} following manufacturer's instructions.

The relative transcript level determination was performed by quantitative PCR in triplicate using SYBR Green PCR Master Mix (Applied Biosystems). Primers pairs used are: *Synapsin I* (fwd: 5'- AGCTCAACAAATCCCAGTCTCT-3', rv: 5'- CGGATGGTCTCAGCTTTCAC- 3'); *Neuroglycan C* (fwd: 5'- TCTTCCTCACATCTCCCCTTTT - 3', rv: 5'- TCTTCAAAGCTGTTTGGTGACTG - 3'); *β3-Tubulin* (fwd: 5'- TAGACCCCAGCGGCAACTAT - 3', rv: 5'- GTTCCAGGTTCCAAGTCCACC - 3'). The housekeeping gene used as endogenous normalizer was *Gadph* (fwd: 5'- AGGTCGGTGTGAACGGATTTG – 3', rv: 5'- TGTAGACCATGTAGTTGAGGTCA - 3'). Thermocycling conditions were 10 min at 95°C, then 40 cycles of 15 sec at 95°C and 1 min at 60°C. The program was performed by QuantStudio 5 Real-Time PCR System (Applied Biosystem). The obtained Ct values were normalized to the respective value of *Gadph* and expressed as a function of the control condition (untreated neurons) using the comparative Ct method (ΔΔCt), considering PCR efficiency (Livak *et al.* 2001).

9. Gangliosides analysis

For the analysis of endogenous ganglioside pattern, neurons treated or not with 50 µM oligoGM1 were lysed in water and subjected to lyophilization. Total lipids were extracted from lyophilized cells with the solvent mixture of chloroform/methanol/ water, 2:1:0.1, by vol, followed by a second and third extraction with chloroform/methanol, 2:1 by vol (Chiricozzi *et al*. 2014; Samarani *et al*. 2018; Malekkou *et al*. 2018). The total lipid extracts

were then subjected to a two phase partitioning, by adding 20% of water to the lipid extract resulting in the separation of an aqueous phase containing gangliosides and an organic phase containing all other lipids (Folch *et al*. 1957). Following a dialysis step to remove salts, gangliosides contained in the dialyzes aqueous phase, corresponding to 700 µg of proteins, were separated by a first run in chloroform/methanol 2/1 (v/v), followed by a second run in chloroform/methanol/ 0.2% aqueous calcium chloride 50/42/11 (v/v/v). Following solvent evaporation, gangliosides were revealed by specific detection spraying with Anisaldehyde reagent, and heating at 120° C for 15 min; co-migration with standards allowed the identification of gangliosides species. The relative amount of each ganglioside, was determined by densitometry using ImageJ software (NIH, Bethesda, USA; http://rsbweb.nih.gov/ij/).

9.1 Time course analysis of gangliosides by Ctx-B-HRP staining

The expression of complex gangliosides over time was followed by the detection technique which uses the subunit B of cholera toxin conjugated to peroxidase. This subunit binds GM1 specifically and has often been used to identify GM1 in cell cultures and on TLC plates after separation of gangliosides, together with more complex gangliosides following the reaction with sialidase (Chiricozzi *et al*. 2018). In this way, the levels of GM1, GD1a, GD1b and GT1b could be analyzed. Ctx-B-HRP staining is an extremely sensitive technique, capable of identifying and quantifying even a few picomoles of GM1 ganglioside (Wu *et al.* 1988). To specifically detect GM1, GD1a, GD1b and GT1b gangliosides' content in CGN treated or not with oligoGM1, gangliosides of aqueous phase, equivalent to 15 µg of proteins, and standard of gangliosides, equivalent to 10 pmol, were spotted on TLC plate and subjected to a first-run in chloroform/methanol 2/1 (v/v) followed by a second run in chloroform/methanol/0.2% aqueous calcium chloride 50/42/11 (v/v/v). Following solvent evaporation, the plate was treated for 30 sec with a solution of 0.2% polyisobutylmethacrilate in hexane (v/v) for three times and air dried for 1 h. After 30 min of pre-incubation at 23°C in 1M Tris-HCl pH 8, 0.14 M NaCl containing 1% BSA (w/v), the plate was subjected to the reaction with sialidase diluted at 0.12 U/mL in 0.05 M acetate buffer (pH 5.4), 4 mM $CaCl_2$, for 2 h at 37°C. Then the plate was overlaid with Ctx-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-phenylenediamine (1 tablet in 12.8 mL of citrate-phosphate buffer 0.05 M (pH 5) and 17 µL of H_2O_2. The amount of GM1, GD1a, GD1b and GT1b was calculated by comparing the intensity of the spot in the different samples with respect to that of the

standard equivalent to 10 pmol by densitometry using ImageJ software (NIH, Bethesda, USA; http://rsbweb.nih.gov/ij/).

10. Statistical analysis

Data are expressed as mean ± SEM and were analyzed for significance by Student's t-test or two-way ANOVA test. The analysis was performed with Prism software (GraphPad Software, Inc. La Jolla, CA, USA).

11. 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).

Results

Effect of GM1/GD1a and their oligosaccharides on neuronal morphology

It has been known for long time that exogenously administered GM1 induces the differentiation of murine N2a neuroblastoma cells and it has recently been observed that the administration of the oligosaccharide component alone induces the same neuritogenic effect, demonstrating that GM1 sugar is responsible for this effect (Chiricozzi *et al*. 2017). We therefore investigated the effect of exogenous administration of GM1 and its more complex catabolic precursor GD1a, on the morphology of CGN, adding them to the culture medium from time zero at the final concentration of 50 μM. Figure R1 shows the representative images of optical microscopy acquired after 24, 48 and 72 h of the CGN grown in the absence (CTRL, first line) or in the presence of GM1 (GM1, second line) or GD1a (GD1a, third line), which show that both gangliosides seem to accelerate the process of differentiation of granular progenitors by speeding up clustering and the emission of neuronal prolongations.

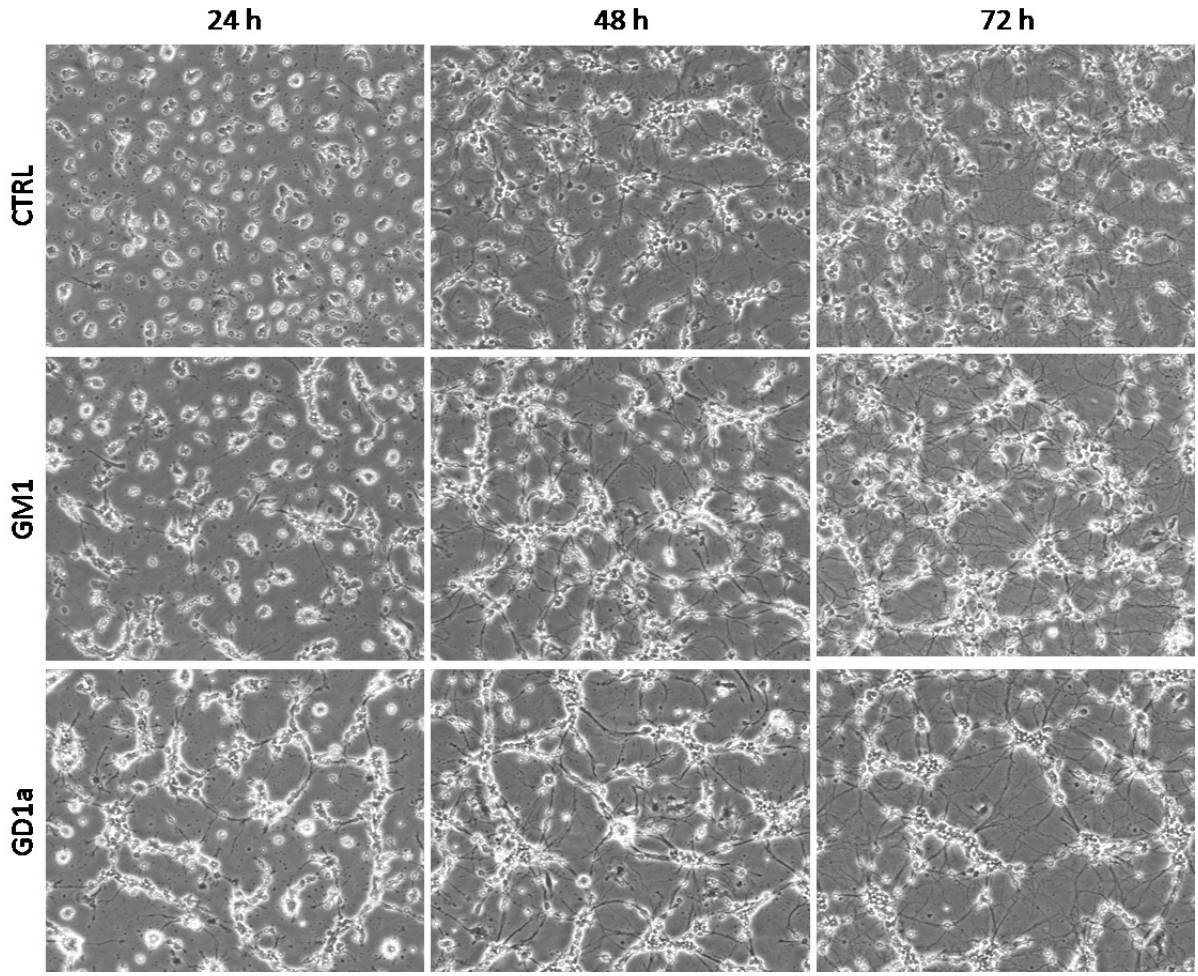


Figure 1R. Morphological effect of exogenous administration of gangliosides GM1 and GD1a to cerebellar granule neurons during development. CGN were dissociated from mice cerebellum and plated in the absence (CTRL, first line) or in the presence of 50 μM of GM1 (second line) or of GD1a (third line). Representative phase contrast microscopy images with 100 x magnification obtained after 24, 48 and 72 h from cell plating (*n*=8) are reported.

At this point we investigated whether analogue morphological effect occurred following the administration of the respective oligosaccharide chains of GM1 (oligoGM1) and GD1a (oligoGD1a). Figure R2 shows the representative images of optical microscopy of cerebellar granular progenitors grown in the absence (CTRL, first line) or in the presence of oligoGM1 (oligoGM1, second line) or oligoGD1a (OligoGD1a, third line) added to the culture medium at time zero at a concentration of 50 μM.

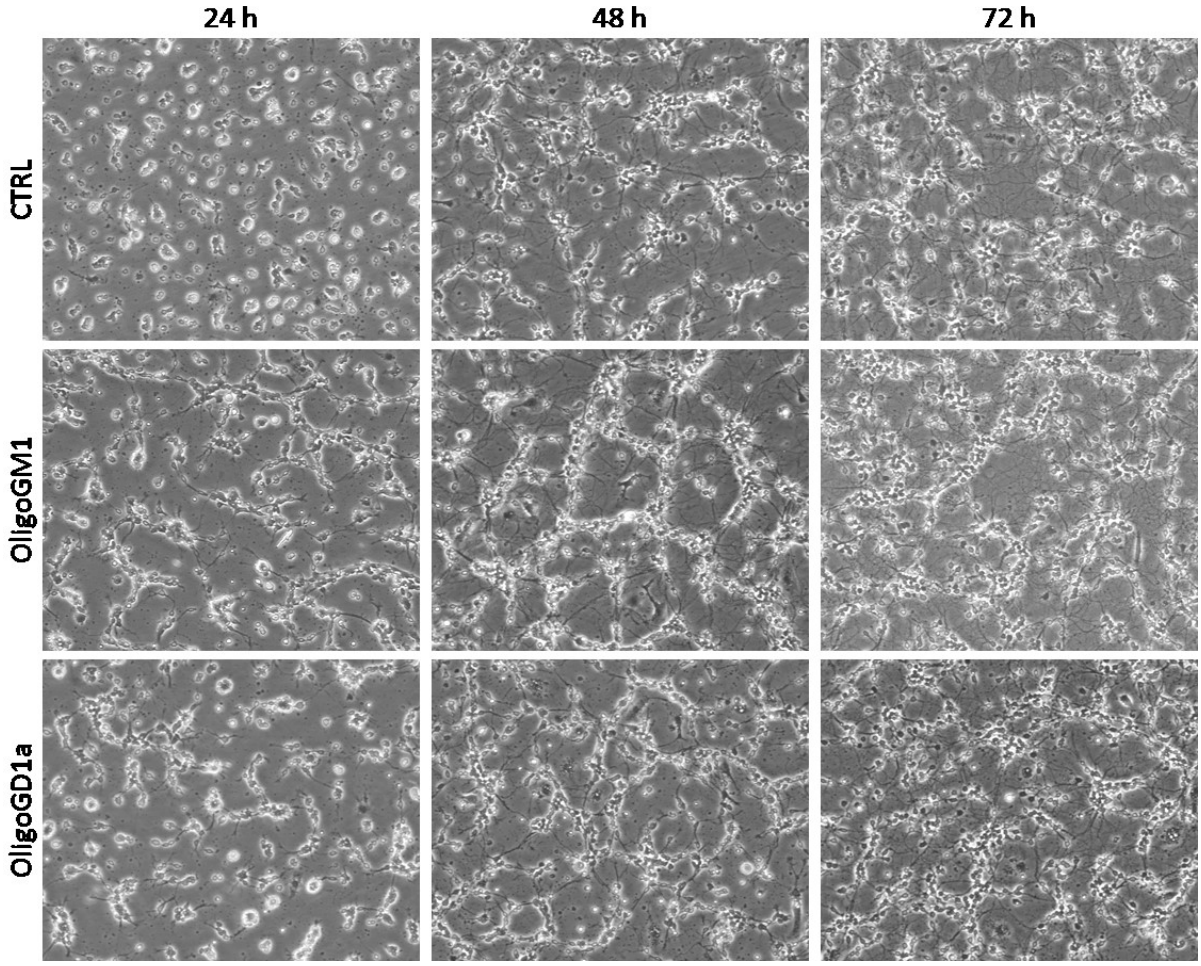


Figure 2R. Morphological effect of exogenous administration of gangliosides oligosaccharides oligoGM1 and oligoGD1a to cerebellar granule neurons during development. CGN were dissociated from mice cerebellum and plated in the absence (CTRL, first line) or in the presence of 50 μM oligoGM1 (second line) or oligoGD1a (third line). Representative phase contrast microscopy images with 100 x magnification obtained after 24, 48 and 72 h from cell plating (*n*=8) are reported.

In this case only the oligoGM1 has shown to induce an acceleration of the clustering and the neuritogenesis of the CGN with respect to the untreated control cells, while the cells grown in the presence of oligoGD1a seem to follow maturation times analogous to the control cells. We also performed an immunofluorescence analysis on cells grown in the absence or in the presence of oligoGM1 using antibodies directed against neurofilament proteins, which characterize neuronal extensions and increase during neuron maturation, allowing to follow the organization and the neuronal disposition in culture. As shown in Figure R3, the CGN show longer extensions and a more complex arrangement after 48 h incubation with oligoGM1 with respect to controls. The DAPI staining of the nuclei shows a more organized disposition of the cell bodies in oligoGM1-cells, with an accentuated degree of aggregation.

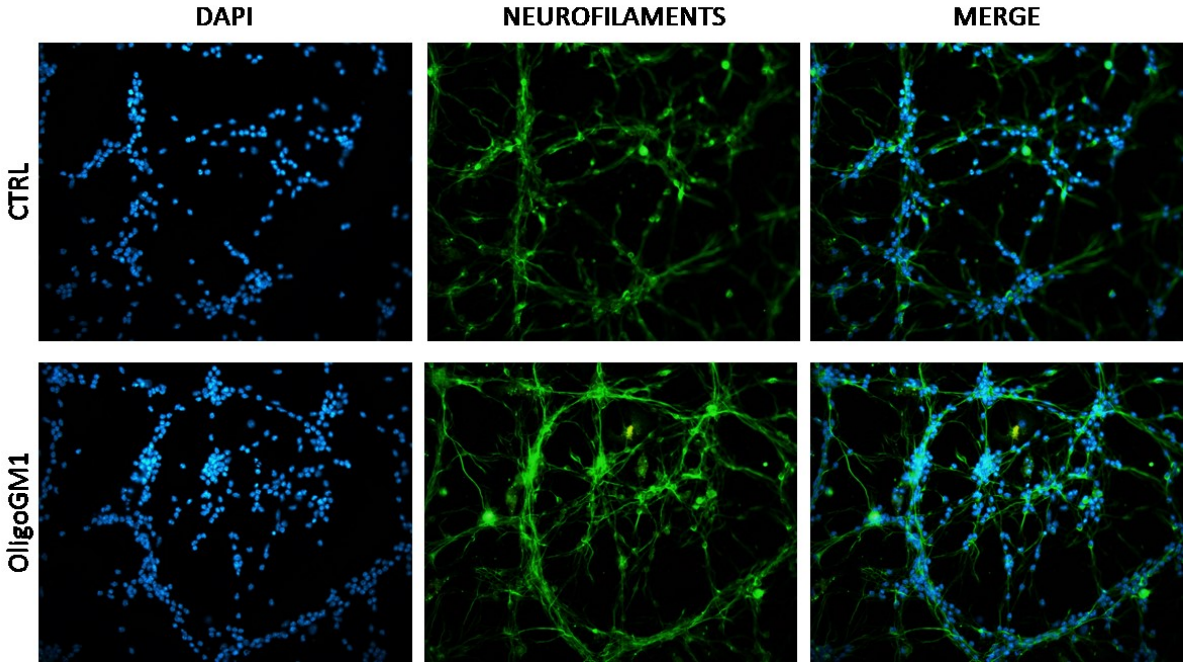


Figure 3R. Immunofluorescence analysis of Neurofilament proteins in CGN under oligoGM1 administration. Representative immunofluorescence images with 100x magnification for nuclei (DAPI - blue), Neurofilament proteins (green), and merged signal (MERGE) following 48 h from neurons plating (*n*=3).

Hence, in the presence of oligoGM1 as well as of GM1 and GD1a, CGN show typical clusters already following 24 h in culture and more complex neurite network compared to cells grown with oligoGD1a or untreated ones.

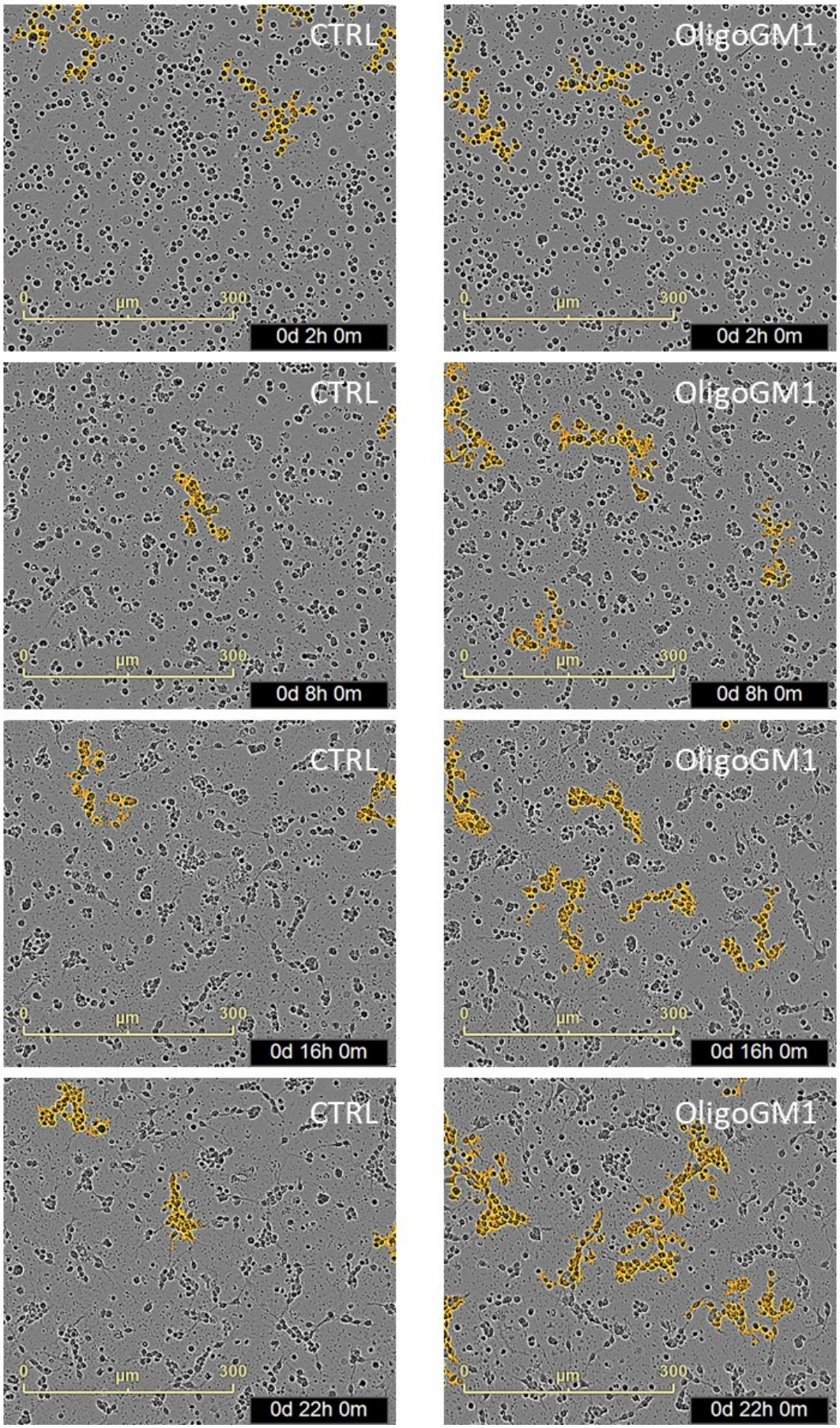
Even in primary neurons, the oligosaccharide component of GM1 appears to be responsible for the morphological effects of the entire GM1 and of its precursor GD1a. For this reason,

we focused on oligoGM1 characterizing its effect on neuronal motility and the underlying mechanism of action.

Effect of oligoGM1 on neuronal motility

To better investigate the influence of oligoGM1 on neuronal motility, time lapse recordings of neuronal cultures in the presence and absence of oligoGM1 using the Incucyte® Live Cell Analysis System (Sartorius) were performed. The frames were acquired every 2 h for 24 h. Video cuttings representing the acquired fields are shown in Figure 4R (panel A). The graph reported in Figure 4R (panel B) shows that the cells incubated with oligoGM1 have a significantly higher degree of clustering than controls starting from 8 h in culture and lasting for all 24 h.

A



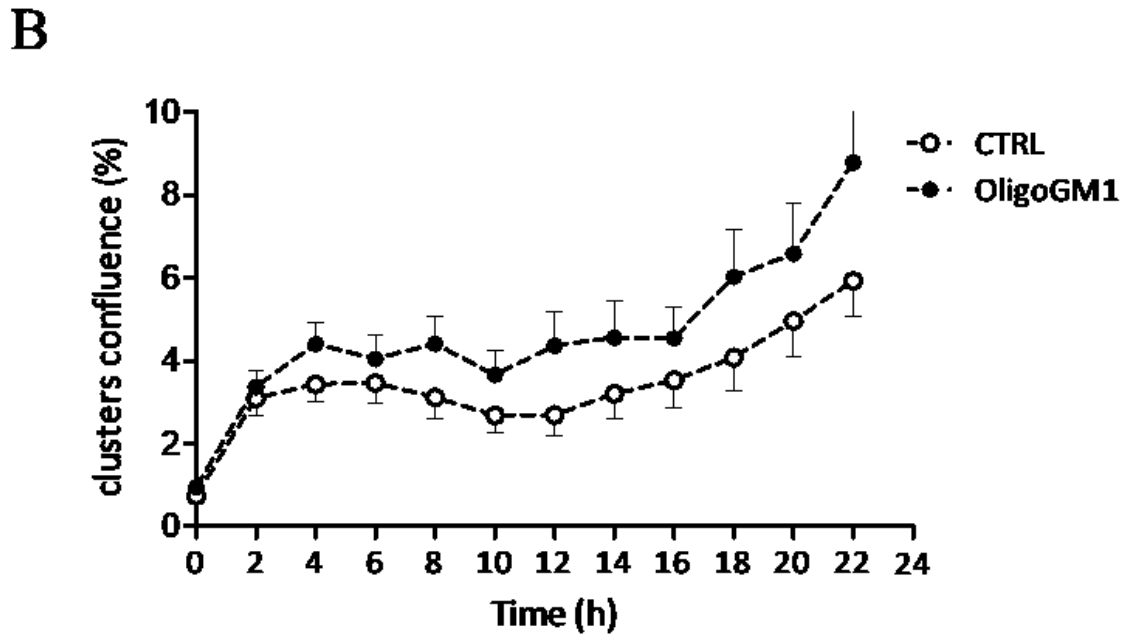


Figure 4R OligoGM1 effect on neuron clustering. The CGN were plated with or without 50 μM oligoGM1 and in-live recorded acquiring 1 frame every 2 hours for 24 hours via IncuCyte. a) Time lapse frame cuttings acquired with a magnification of 100 X at the times indicated. The clusters automatically defined by the IncuCyte S3 2018A software are highlighted in yellow. b) Cell clustering rate quantification carried out by IncuCyte S3 2018A software. White circles represent neurons cultured in the absence of oligoGM1 and black circles represent neurons grown in oligoGM1 supplemented medium. Results are expressed as the mean ± SEM of cluster confluence percentage from three different experiments (*$p<0.05$ vs. CTRL, two-way ANOVA, $n=3$).

OligoGM1 impact on focal adhesion protein activation

To biochemically characterize the effect of oligoGM1 on neuronal migration, we investigated the involvement of focal adhesion proteins. FAK is activated by several extracellular stimuli such as those arising from adhesion receptors (integrins) and receptors for neurotrophins. The active form of FAK is phosphorylated on tyrosine 397 and in primary neurons it was found to form a complex with the Src kinase, activated in turn by phosphorylation. (Tucker *et al.* 2008; Carragher *et al*. 2004). In this way FAK and Src recruit a series of proteins involved in the actinic cytoskeletal remodeling, necessary to allow motility and growth of the axonal cone. Among these proteins there are, for instance, paxillin, vinculin and the small GTPases proteins.

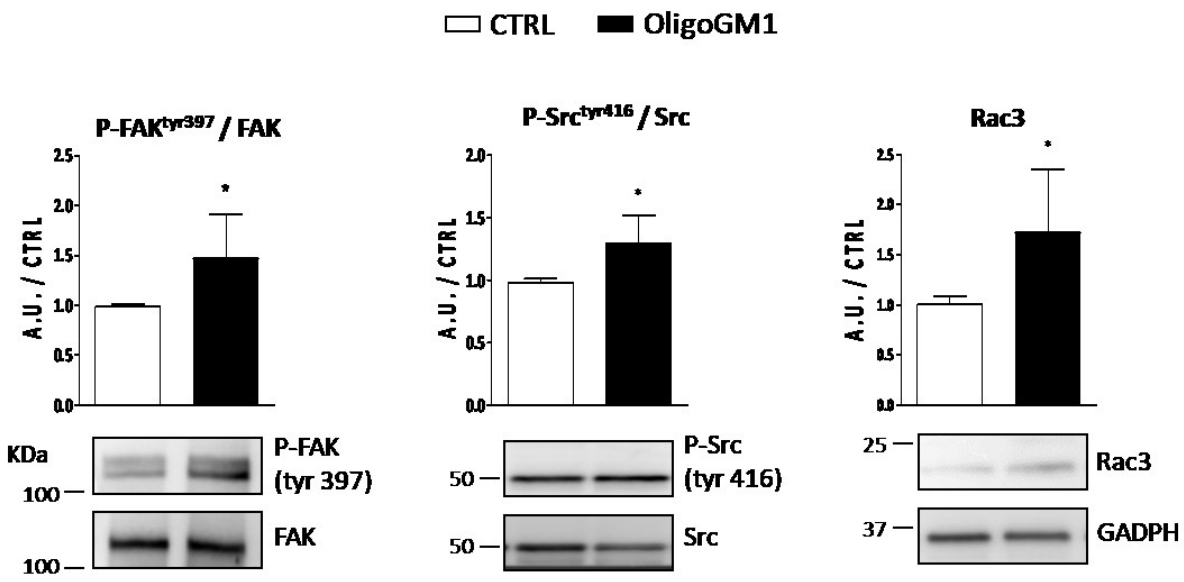


Figure 5R OligoGM1 effect on the expression and activation of focal adhesion proteins. CGN were incubated in the absence (CTRL) or in the presence of 50 μM oligoGM1 for 24 h. Expression of FAK, P-FAK (tyr 397), Src, P-Src (tyr 416), and RAC3 were carried out by specific antibodies and signals were revealed by enhanced chemiluminescence. Bottom: representative immunoblotting images are shown. Top: semi-quantitative analysis of P-FAK and P-Src signals related to total level of FAK and Src, respectively; RAC3 was normalized on GADPH level, used as internal normalizer. Data are expressed as fold increase over control of the mean ± SEM from five different experiments (*$p<0.05$, Student's t-test, $n=5$).

As shown by immunoblotting represented in Figure 5R, after 24 h from oligoGM1 administration, an increased phosphorylation levels of both FAK and Src kinases was found. It was reported that during neuronal development the expression of Rac3 protein, a small GTPase belonging to Rho-GTPase family, is specifically induced to regulate neuritogenesis and neuron migration (Albertinazzi *et al.* 1998; Corbetta *et al*. 2009). Interestingly, an increased level of Rac3-GTPase protein in CGN cells supplemented with oligoGM1 with respect to controls following 24 h of plating was pointed out (Figure 5R).

OligoGM1 influence on the expression of neuronal marker proteins

During neuronal differentiation, development and maturation, there is a significant change in the expression of specific genes coding for structural proteins and enzymes required for proper neuronal function. The effect of oligoGM1 on the protein pattern of cultured neurons was evaluated, investigating neuronal markers whose expression progressively increase during neuritogenesis and maturation such as Neuroglycan C and β3 tubulin, which are

constituents of neuronal protrusions, and Synapsin I which is part of the synaptic density (Aureli *et al*. 2011; Ngamukote *et al.* 2007).

As shown in the Figure 6R (panel A) following 24 h from oligoGM1 administration, CGN have been found to express increased protein level of all tested markers with respect to untreated neurons.

In parallel the mRNA level of the aforementioned markers was verified, using quantitative PCR. RNA was extracted and retrotranscribed from neuronal lysates. The graphs reported in Figure 6R (panel B) show that the transcript level of neuronal markers undergoes a significantly increase in the CGN after 5 h of incubation with oligoGM1 compared to the untreated controls. After 12 and 24 h in culture, the mRNA levels of the markers remain higher in the oligoGM1 treated CGN compared to the controls, but at a lower extent.

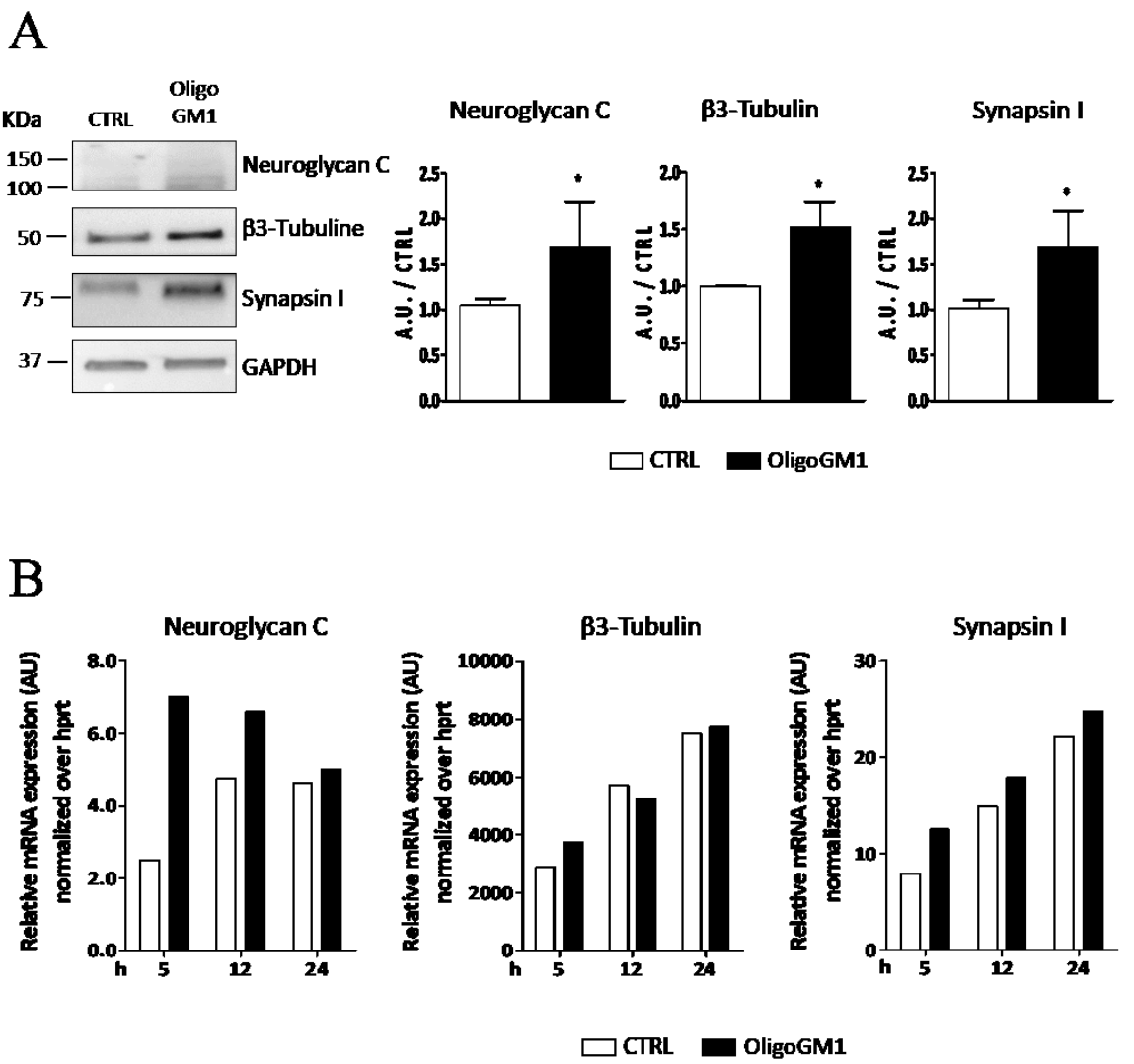


Figure 6R OligoGM1 effect on the expression of neuronal markers. CGN were plated in the absence (CTRL) or in the presence of 50 μM oligoGM1. The expression of Neuroglycan C, β3-tubuline and Synapsine I was evaluated both at protein and at transcript level. a) Western blotting analysis of cell lysate after 24 h from cell plating by means of specific antibodies and revealed by enhanced chemiluminescence. GADPH was used as internal normalizer. On the left, representative immunoblotting images are shown. On the right, relative quantitation of protein signals is reported. Data are expressed as the mean ± SEM of fold increase over control from five different experiments (*p<0.05, Student's t-test, *n*=5). b) RT-PCR after 5, 12 and 24 h from cell plating. Hprt gene was used as internal normalizer. The relative expression level is indicated as mean ± SEM of three different experiments (Student's t, *n*=3).

Dynamics of the ganglioside pattern under the influence of oligoGM1

Glycosphingolipids and their sialic acid-containing derivatives, gangliosides, are important cellular components and are abundant in the nervous system. They are known to undergo dramatic changes during brain development. Gangliosides are particularly represented in the

membrane of neurons. During differentiation and maturation *in vitro*, as well as *in vivo*, the composition of neurons' gangliosides changes drastically, both to define the peculiar membrane geometry of the synapses, and to constitute the important signaling platforms characterized by the enrichment of gangliosides, i.e. lipid rafts. The simplest gangliosides such as GM3 and GD3 are more represented in neuronal precursors, where instead more complex gangliosides are less expressed. During maturation and neurites extension, complex gangliosides such as GM1, GD1a, GD1b and GT1b increase progressively and their levels remain high until senescence (Van Echten-Deckert *et al*. 2006; Aureli *et al*. 2011). In mature neurons, on the other hand, the quantity of simpler gangliosides, especially GM3, is reduced. Therefore, the possible effect of exogenous administration of oligoGM1 on the dynamics of the ganglioside pattern of CGNs was evaluated, focusing on the time of 24 h of culture, where an effect of GM1 oligosaccharide on the expression of neuronal protein markers was detected.

The cells were plated directly in the presence or absence of oligoGM1 and were collected and lysed after 24 h. The lipids were then extracted as in the Materials and Methods section and the gangliosides were separated from the neutral lipids by the Folch partitioning (Folch *et al*. 1957) within the aqueous phase. The different species of gangliosides were separated by hpTLC and revealed with the Anisaldehyde reagent. A representative image of the hpTLC plate is shown in Figure 7R. The intensity of each band, corresponding to the specific lipid species, has been normalized with respect to the protein content in the samples and compared to the levels of the control. The graph on the right shows the quantification of gangliosides where it is highlighted that in the presence of oligoGM1 CGN express higher levels of GM1 ganglioside while GM3 is slightly less expressed.

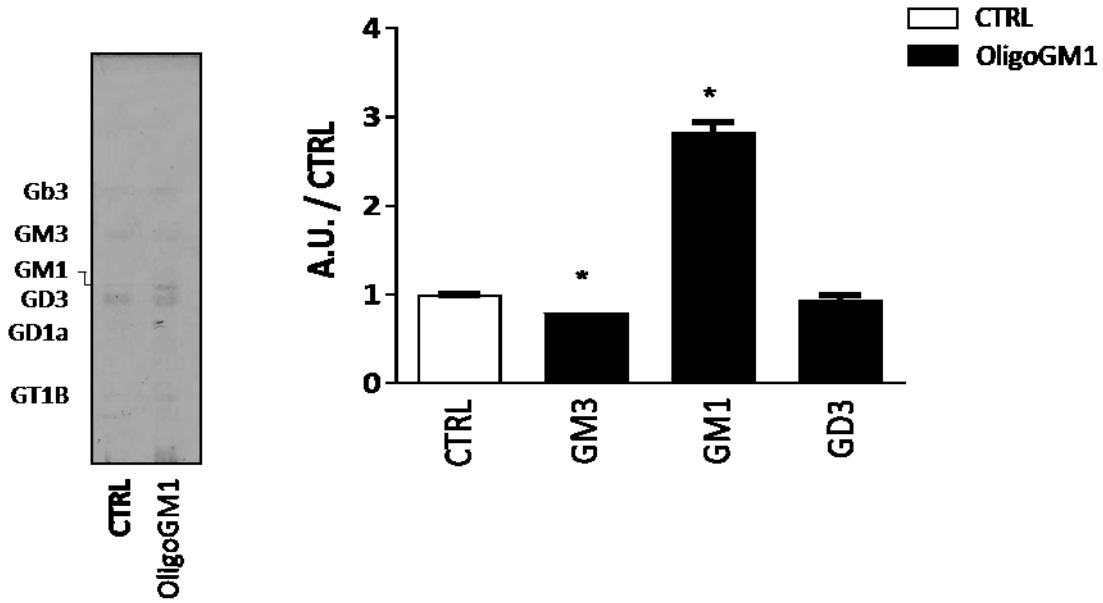


Figure 7R. OligoGM1 effect on CGN endogenous gangliosides. The endogenous gangliosides were separated from total lipid extract obtained from neurons cultured in the absence (CTRL) or in the presence of 50 μM oligoGM1 for 24 h. Ganglioside pattern resolved by hpTLC in the presence of specific standards and revealed by anisaldehyde spry reactive. On the left: hpTLC plate representative image of neuron ganglioside pattern. On the right: relative quantitation of the intensity of each ganglioside band normalized on the protein content. Data are expressed as the mean ± SEM of fold increase over control from three different experiments (*$p<0.05$, Student's t-test, $n=3$).

In addition, we followed the expression of complex gangliosides over time, by using Ctx-B-HPR as detection technique. The Figure 8R reports the results of the Ctx-B-HRP staining analysis following treatment with sialidase on TLC plate, performed after separation of the gangliosides extracted from CGN grown in the presence or in the absence of oligoGM1 for 12, 24, 48 and 72 h. The graphs on the bottom show the quantification of each lipid species normalized to protein content. Already starting from 12 h in culture the CGN treated with oligoGM1 show greater levels of complex gangliosides which remain higher than the controls even after 72 h.

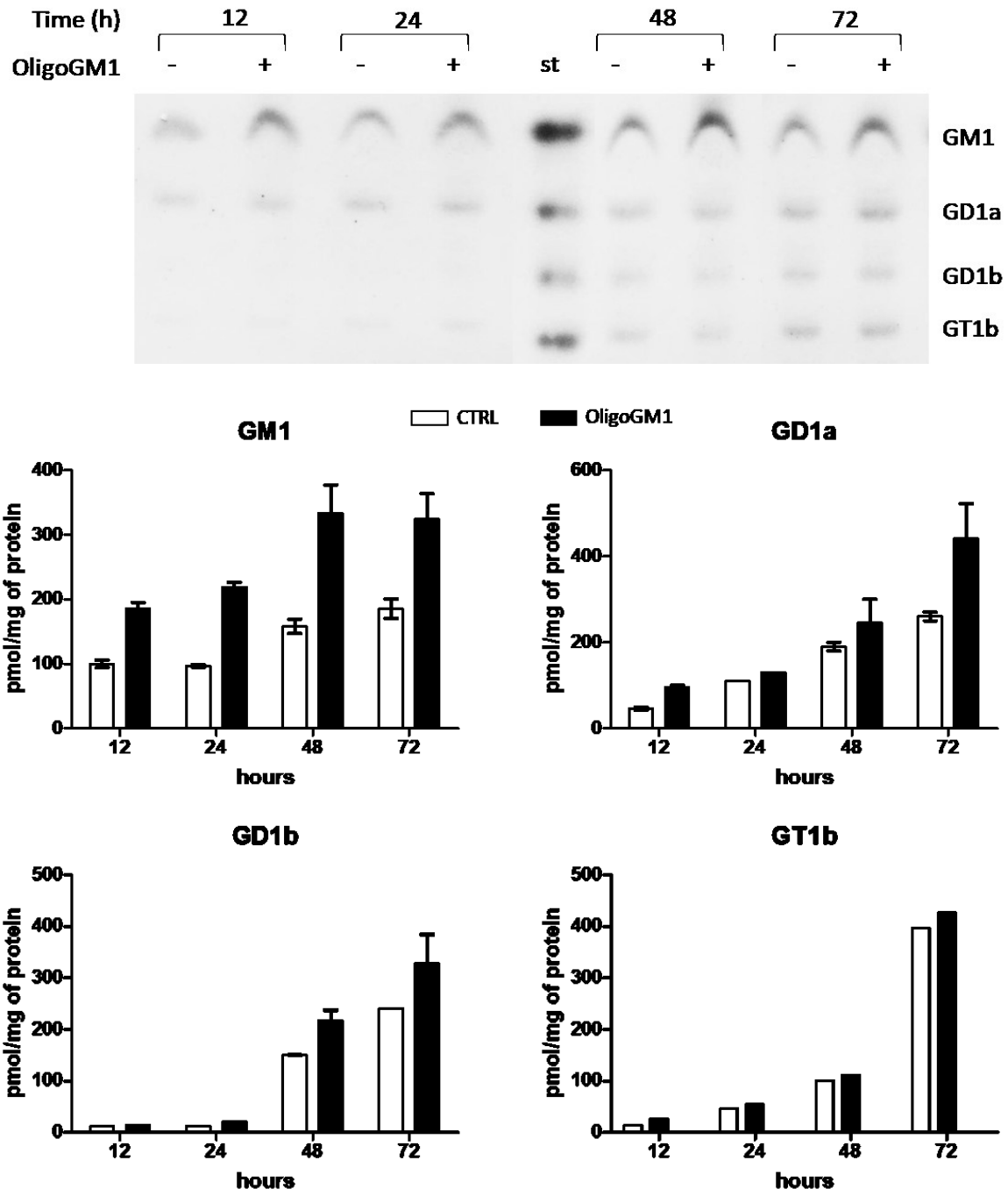


Figure 8R Time course effect of oligoGM1 on the CGN ganglioside composition detected by Ctx-B-HRP staining. The CGN were plated in the absence (CTRL) or in the presence of oligoGM1. After 12, 24, 48 and 72 h the lipids were extracted, and the gangliosides separated as described in the Methods section. The ganglioside species were resolved by hpTLC in the presence of defined standards determined of 10 pmol and detected by staining with Ctx-B-HRP following reaction with sialidase on the hpTLC plate. On the top, the representative image of the hpTLC plate. Below are the graphs of the quantifications of the levels of each lipid expressed as mean ± SEM of pmol per mg of total proteins from three different experiments (n = 3, Student T test, CTRL vs oligoGM1, $p < 0.05$).

Fate of [^{3}H]-oligoGM1 added to primary neurons

Recently, in neuroblastoma cell line N2a, oligoGM1 was found to activate differentiating program by interacting with cell surface without entering into the cells (Chiricozzi *et al*. 2017).

In order to characterize the molecular mechanism underlying oligoGM1 effect also in primary neurons, we investigated how oligoGM1 could interact with cultured CGN following the procedure previously used (Chigorno *et al*. 1985; Chiricozzi *et al.* 2017). CGN were cultured in the presence of isotopic tritium labeled [^{3}H]-oligoGM1 for 0.5, 1, 4, 6, 12 and 24 h. At the end of the incubation, neurons were firstly washed with culture medium containing 10% serum in order to collect the fraction of oligosaccharide weakly associated to the cells' surface; this one represented the serum labile fraction. Subsequently cells were washed with a solution containing low concentrated trypsin to remove the possible fraction strongly bond to the cell surface, corresponding to the trypsin labile fraction. At the end, cells were lysed obtaining the fraction of internalized [^{3}H]-oligoGM1 corresponding to the trypsin stabile fraction. The graph in Figure 9R shows the radioactivity values measured in each fraction for all the times considered. At each time point analyzed, about 99% of [^{3}H]-oligoGM1 was found in the serum labile form suggesting that the oligoGM1 is not taken up by the cells but rather it weakly associate to neuron plasma membrane.

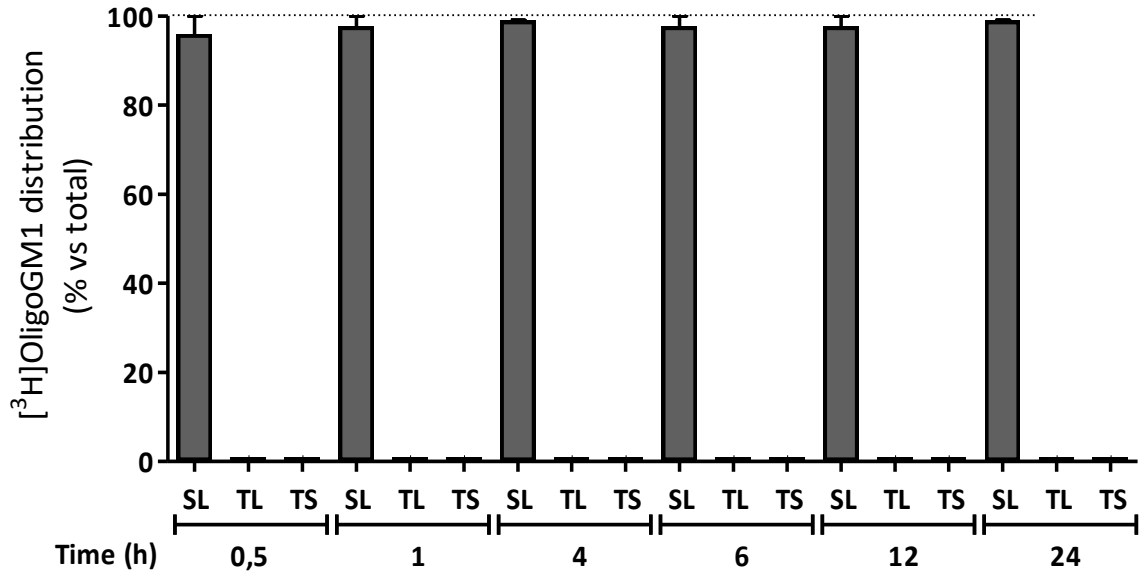


Figure 9R Association of [3H]-oligoGM1 to CGN. CGN were incubated with 50 μM [^{3}H]-oligoGM1 for 0.5, 1, 4, 6, 12 and 24 h. After pulse, cells were washed with medium containing 10% FBS to obtain the serum labile fraction (SL). Then cells were washed with low concentrated trypsin solution to obtain the trypsin labile fraction (TL). Finally, cells were lysed to obtain trypsin stable fraction (TS) corresponding to the fraction of compound internalized by cells. The radioactivity associated with each fraction was determined by liquid scintillation counting. Data are expressed as mean ± SEM of total radioactivity percentage from three different experiment (*n*=3).

TrkA-MAPK pathway as the mediator of oligoGM1 prompted neuronal maturation

In primary neurons, as well as in N2a neuroblastoma lines, the oligoGM1 is not internalized by the cells, so its effects could probably be derived by an action at the level of the plasma membrane. Furthermore, taking into account the reported effect prompted by GM1 on TrkA mediated neuronal differentiation (Farooqui *et al*. 1997; Singleton *et al*. 2000, Duchemin *et al.* 2002; Da Silva *et al.* 2005; Mocchetti 2005; Zakharova *et al*. 2014) and considering the recent result in neuron-like N2a cells, where oligoGM1 was found to increase TrkA phosphorylation (Chiricozzi *et al.* 2017, 2019a, 2019b), we investigated the activation of the TrkA-MAPK pathway with the aim of identifying the molecular effectors of oligoGM1 in CGN.

Thus, we performed a time course analysis collecting the neurons after a few minutes up to 3 h following oligoGM1 administration and evaluating, by western blotting, the phosphorylation status of the TrkA receptor and MAPK ERK1/2.

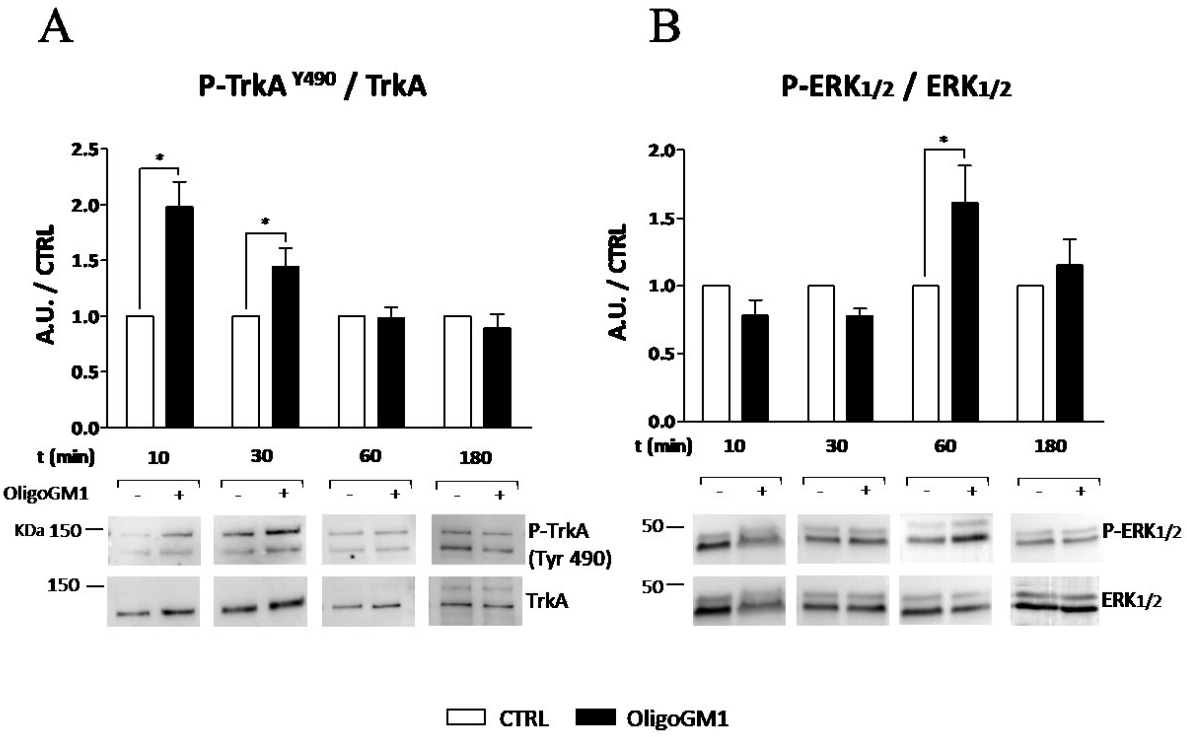


Figure 10R OligoGM1 effect on TrkA-ERK1/2 pathway. CGN were culture in the absence (CTRL) or in the presence of 50 μM oligoGM1. Expression of TrkA, P-TrkA (tyr490), ERK1/2 and P-ERK1/2 by specific antibodies and signals were revealed by enhanced chemiluminescence. Top: representative immunoblotting images. Bottom: semi-quantitative analysis of spots' signals of phosphorylated TrkA and ERK1/2 related to signals of total TrkA and ERK1/2, respectively. Data are expressed as mean ± SEM of the fold increase over control from five experiments (*$p<0.05$, Student's t-test, $n=5$).

TrkA activation

As reported in the blotting of Figure 10R (panel A) we observed a hyperphosphorylation of TrkA on tyrosine 490 after only 10 min from the administration of oligoGM1 which remains higher than the control cells for 30 min. This difference disappears 1 h after sugar administration.

ERK1/2 pathway

After 10 and 30 min from administration of oligoGM1, no difference in ERK1/2 phosphorylation was seen in treated neurons compared to controls. Only after 1 h the

oligoGM1 treated neurons had a higher level of ERK1/2 phosphorylation than the controls, which returned to equalize the untreated neurons after 3 h.

Discussion

The role of gangliosides and specifically GM1, in nervous system homeostasis has been known for several decades and many GM1-modulated molecular partners have been identified over the years. Moreover, in 2017 a research hypothesis, advanced in 1988 by Shengrund and Prouty (Shengrund and Prouty 1988), was resumed according to which an essential role for GM1 to carry out its plasma membrane mediated functions should be attributed to its oligosaccharide portion (Chiricozzi *et al*, 2017). The exploration of GM1 oligosaccharide functionalities was performed in neuron-like cells, i.e. murine N2a neuroblastoma cells, where it was shown that the pentasaccharide (β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)-]β-Gal-(1-4)-Glc, coded $II^3Neu5Ac\text{-}Gg_4$) was the only bioactive component of GM1 ganglioside, responsible for its neuritogenic and protective properties (Chiricozzi *et al*, 2017; 2019a; 2019b).

Here, therefore these findings were translated in a more physiological context by using primary cultures of precursors of murine cerebellar neurons, with the aim to dissect the GM1 molecular mechanism regarding the differentiation processes.

To do that, the potential effects of GM1, its direct anabolic successor/catabolic precursor GD1a, and their oligosaccharides where exanimated on the process of neuronal differentiation and maturation *in vitro*.

Both GM1 and GD1a gangliosides applied induced an acceleration of the development of neurons, which can be deduced from a more rapid formation of neuronal clusters, which corresponds to the first phase of the maturation process of these neurons *in vitro* (Bilimoria *et al.* 2008) (Figure 1R). It should be noted that this effect occurred starting from the first hours of culture, where the cell body clusters were visibly more numerous and populated in the presence of gangliosides and continued until the third day where the differences between cells incubated with and without gangliosides were smoothed. The same morphological effect was observed when GM1-oligosaccharide was administered, whereas the GD1a-oligosaccharide administration provided no difference recorded with respect to the control cells (Figure 2R). This suggests that even in primary neurons the oligoGM1 accounts for the effect of the whole GM1, while the oligoGD1a does not account for the effect of the whole GD1a. As with GM1, when GD1a is administered exogenously, it is taken up by cells and becomes an additional component of plasma membranes (Saqr *et al*. 1993). It is possible to speculate that the exogenously administered and stably inserted GD1a in the neuron membrane is converted to GM1 by cellular plasma membrane sialidases (Miyagi *et al*. 2012). In fact, GD1a is known to cluster with GM1 and sialidases with a role of reserve pool of GM1 (Sonnino *et al*. 2011). In this way the morphological effect observed following the

administration of GD1a could be ascribed to the GM1 deriving from its desialylation and more likely to GM1-oligosaccharide component since GD1a-oligosaccharide alone is not able to exert the same effect.

Since as in N2a cells, oligoGM1 exerted a morphological effect similar to that of GM1 (and GD1a), we focused our subsequent analyzes only to oligoGM1, to fully characterize the GM1 mechanism of action.

OligoGM1 influence on neuron migration has been analyzed in more detail through live imaging. The time lapse analysis recorded a significant increase of neuron-clustering rate in the presence of oligoGM1 starting from 8 h in culture which remained higher than the controls for all the 24 h of analysis (Figure 4R). The increased aggregation state is observed even after 48 h where the immunofluorescence for neurofilament proteins has also highlighted a more complex neuronal network formed by the CGN grown in the presence of the oligoGM1 with respect to the controls (Figure 3R).

The biochemical explanation of this morphological effect is revealed by the increase in the pFAK(Y397)/FAK ratio evaluated by immunodetection, in oligoGM1 grown cells, accompanied by the elevation of the pSrc(Y416)/Src ratio (Figure 5R). Accordingly, in 2014, Navarro and co-workers defined the focal adhesion kinase FAK functioning as a sort of "orchestra director" that coordinates a wide range of signaling pathways during neuronal motility and growth of axons and dendrites (Navarro and Rico 2014). In fact, FAK associates with and activates the kinases of the Src family and in combination they recruit an array of other proteins involved in the actin cytoskeletal remodeling, an event required both for the advancement of the leading edge of a lamellipodium, and for the growth of neuritic prolongations (Tucker *et al*. 2008; Carragher *et al*. 2004).

In addition, the observation of increased levels of Rac3-GTPase in CGN grown in the presence of oligoGM1 further supports the role of the saccharide in stimulating neuron migration during the early stages of development as Rac3-GTPase is reported to be specifically expressed during the early stages of neuronal migration (Bolis *et al*. 2003; De Curtis *et al*. 2014).

Beyond the migratory aspect, we found that oligoGM1 accelerated all changes in the protein and lipid pattern that normally occur during the process of neuronal differentiation and maturation. After 24 h the CGN expressed higher levels of specific neuronal protein markers when cultivated in the presence of oligoGM1 with an increase in the respective transcript levels observable already 5 h after the saccharide administration (Figure 6R). Furthermore, even the shift from simplest gangliosides towards the expression of more complex

gangliosides was accelerated by oligoGM1 (Figure 7R). During neuronal differentiation and complete maturation, neurons reduce the expression of the simplest gangliosides (GM3, GD3) while the most complex ones increase with GM1, GD1a, GD1b and GT1b representing the main gangliosides belonging to completely differentiated neurons (Yu *et al*. 2008, Prinetti *et al*. 2001; Aureli *et al*. 2011). Already after 12 h in culture in the presence of oligoGM1, the CGN expressed higher levels of the four main complex gangliosides and after 24 h there was a decrease in GM3 levels compared to the control cells (Figure 7R, 8R).
Overall, oligoGM1 appeared to act as a stimulant and enhancer of the mature neuronal phenotype: the exogenous administration of oligoGM1 could anticipate the physiological increase of plasma membrane GM1 and thus driving in advance all the related events due to the presence of GM1.

The discovery of the oligosaccharide associated almost exclusively with the serum-labile fraction, weakly associated with the plasma membrane, indicated that the saccharide remained in the extracellular environment without being internalized by neurons. In fact, no radioactivity associated with oligosaccharide, when used as a radio-labeled derivative, was measured in neuronal lysates (Figure 9R). This datum suggests that, similarly to what was observed in neuroblastoma cells (Chiricozzi *et al*. 2017; 2019a), the oligosaccharide induces the morphological effect acting at the level of the plasma membrane. With the aim of identifying a pathway that could act as a mediator of the effects of oligoGM1 and then initiate the cascade of events at the plasma membrane level, we investigated the involvement of the TrkA-MAPK pathway, already known to be a GM1 target (Farooqui *et al*. 1997; Singleton *et al*. 2000; Duchemin *et al*. 2002; Da Silva *et al.* 2005; Mocchetti *et al.* 2005; Zakharova *et al*. 2014) and found to be up-regulated by oligoGM1 in N2a cells (Chiricozzi *et al*. 2017; 2019b).
Our results indicate a rapid up-phosphorylation of the TrkA receptor, at the level of tyrosine 490, which occurs already 10 min after oligoGM1 administration and persists for another 20 min (Figure 10R, A). Only after 1 h the increase in ERK1/2 phosphorylation is appreciated, whereas at earlier times no significant difference in activation of the ERKs with or without oligoGM1 has been detected (Figure 10R, B). Since ERK is a terminal transducer downstream of a signal transduction cascade that involves other intermediaries in the middle, the delay of the enhanced activation between TrkA and ERK could be due to the presence of the many other factors of the cascade. The interaction between the extracellular portion of TrkA and the GM1 oligosaccharide has already been observed in N2a neuroblastoma cells

and characterized by bioinformatic molecular docking experiments (Chiricozzi *et al*. 2017). It is reasonable to think that the increase in TrkA phosphorylation induced by oligoGM1 in the CGNs is due to a direct association between the two molecules, also in this context.

Interplay between TrkA and activation of FAK, Src and MAPK has already been found in peripheral DRG neurons, where the application of NGF led to the activation of TrkA, FAK, Src and MAPK, events necessary for neuritic outgrowth (Tucker *et al.* 2008). The observation of TrkA-MAPK pathway activation following the administration of oligoGM1 together with the discovery of increased pFAK and pSrc levels suggests that there is an association between the oligoGM1-induced MAPK pathway and the activation of the regulators of focal adhesions, which are directly responsible for neuronal motility and the lengthening of neuronal processes. The morphological differences are accompanied by the activation of cellular signaling processes that finally lead to an acceleration of differentiation processes both from protein and lipid point of view.

The present work demonstrates that the GM1 oligosaccharide is able on its own to exert the same enhancer effect on the neuronal development as GM1 does, confirming what was observed on N2a cells (Chiricozzi *et al*. 2017). The morphological effect basically concerns an acceleration of motility and therefore of the clustering process, together with the strengthening of the neuronal network. As summarized in the diagram of Figure D1, this effect is due to an action at the level of the plasma membrane related to a rapid enhancement of the Trka-MAPK pathway activation. This event is associated with increased activation of FAK and Src, the main regulators of the array of proteins involved in the turnover of focal adhesions, responsible in turn for neuronal mobility and neuritic growth, all accompanied by a parallel increase in proteins and lipids typically associated to neuronal maturation phase.

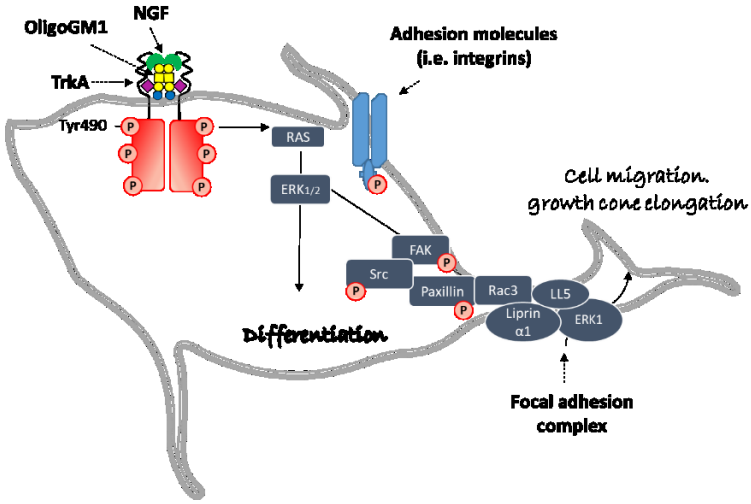


Figure D1. Schematic representation of molecular mechanism underling GM1-oligosaccharide enhanced neuronal maturation. OligoGM1 enhances the activation of TrkA-MAPK pathway which could be associated to an increase of FAK and Src activation, regulators of the array of proteins of focal adhesion complex responsible for neuronal motility and axon growth.

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