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The Crosstalk Between Glycosphingolipids And Neural Stem Cells

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Running Title

Glycosphingolipids and Neural Stem Cells

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Abstract

Until a few years ago, the majority of cell functions were envisioned as the result of protein and DNA activity. The cell membranes were considered as a mere structure of support and/or separation. In the last years, the function of cell membranes has, however, received more attention and their components of lipid nature have also been depicted as important cell mediators and the membrane organization was described

as an important determinant for membrane-anchored proteins activity. In particular, due to their high diversity, glycosphingolipids offer a wide possibility of regulation. Specifically, the role of glycosphingolipids, in the fine-tuning of neuron activity, has recently received deep attention.

For their pivotal role in vertebrate and mammals neural development, neural stem cells regulation is of main interest especially concerning their further functions in neurological pathology progression and treatment. Glycosphingolipids expression present a developmental regulation. In this view, glycosphingolipids can hold an important role in neural stem cells features due to their heterogeneity and their consequent capacity for eclectic interaction with other cell components.

1. Neural stem cells description

During development, neural stem cells (NSCs) play the role of brain formation. This phenomenon persists – although with limited regenerative capacity – throughout postnatal development in the mammalian brain and into adulthood, allowing the continuous generation of new neurons. When Reynolds and Weiss discovered the NSCs in adult mammals brain in the early 1990s (Reynolds & Weiss 1992), they predicted that the impact of their discovery would be substantial: nervous tissue had always been considered invariable (during the adult phase) since the time of Santiago Ramón y Cajal. He wrote in 1913 "*Once development was ended…in the adult centers, the nerve paths are something fixed and immutable. Everything may die, nothing may be regenerated*". (Ramon Y Cajal, S. 1913). This dogma held sway for almost a century. Initially, it was applied to all animals, but later it was discovered that invertebrates were not included, especially during metamorphosis (Truman 1992). Then, lower vertebrates were found to possess regenerative capacity in the central nervous system (CNS) particularly birds during the song learning phase (Alvarez-Buylla et al. 1990). Only at the end of the last century were NSCs found to be present in mammals (Reynolds & Weiss 1992; Bottai et al. 2003; Bottai et al. 2008; Givogri et al. 2008; Adami et al. 2018). From then on, new possibilities of therapeutic approaches were imagined using these cells, hypothesising their use in different neurological pathologies such as spinal cord injury, spinal muscular atrophy, amyotrophic lateral sclerosis, and Parkinson's disease (Bottai et al. 2003;

Bottai et al. 2008; Pluchino et al. 2003; Corti et al. 2007; Daniela et al. 2007; Givogri et al. 2008; Adami et al. 2014).

A large number of studies were performed after their discovery in order to ensure their therapeutical application and to understand the physiology of NSCs.

In common with all other types of stem cells, adult NSCs have a homeostatic function, that is they can replenish the tissue that has lost some of its neural or glial cells due to aging. This phenomenon is more evident in some regions than in others and is more noticeable in lower mammals (mouse) than in humans.

Indeed, it was discovered that the adult mouse brain has three areas that are neurogenic and contain a reservoir of NSCs (Fig. 1): the subventricular zone of the lateral ventricle (SVZ), the sub-granular zone (SGZ) within the hippocampus dentate gyrus and the spinal cord. Adult NSCs in the SGZ generate intermediate progenitor cells (IPCs) (Seri et al. 2001), which go through limited but rapid divisions before they escape the cell cycle and differentiate into neurons and astrocytes. NSCs in adult SVZ may generate neural/glial antigen 2 (NG2) progenitors (that is produced also by other cells such as mesenchymal stem cells (Bottai et al. 2014)), which can originate corpus callosum oligodendrocytes or olfactory bulb interneurons (Bonaguidi et al. 2011). In *in vitro* studies, however, these NSCs are three-potent, producing neurons, oligodendrocytes, and astrocytes.

This homeostatic potential of resident NSCs continues for a long time after birth because these cells have the property of perpetuating themselves (self-renewal) *in vivo* and *in vitro* (Bottai et al. 2003), and of proliferating and differentiating. Moreover, these two last characteristics can be mutually adjusted according to the environment in which they are in contact (Bottai et al. 2003; Pluchino et al. 2003).

In most cases, the definition of NSCs is mostly operative; cells are considered NSCs when they carry all or most characteristics described above. However, a molecular definition is now possible by studying these cells at the protein expression level. Another element of complexity is the fact that, especially *in vivo*, many different types of cells are present in the neurogenic areas.

Two key populations of glial fibrillary acidic protein (GFAP)-positive cells are seen in the SVZ (Jankovski & Sotelo 1996): type B1 and type B2 cells, both displaying attributes of astrocytes (Doetsch et al. 1997). B1 cells are fusiform with two to three processes with few branches (Jankovski & Sotelo 1996); these slowly divide producing transit-amplifying precursors named type C cells with elevated

proliferation rates which then originate migrating neuroblasts (Type A cells), that become interneurons as soon as they arrive in the olfactory bulb (Doetsch et al. 1999). Type B2 cells are called niche astrocytes; they have a stellate aspect similar to parenchymal astrocytes (Lacar et al. 2012) and are located between the SVZ and the striatum (Liu et al. 2005) where they are in contact with blood vessels (Mirzadeh et al. 2008). Another very small population of astrocytes, positive to S100 calcium-binding protein B (S100B), is present in the SVZ in young adult mice (Platel et al. 2009). In humans, half of these S100B positive cells are also NG2 positive (Platel et al. 2009). The cells resident in the SVZ express many different markers as shown in Table 1 where the different cell subtypes in SVZ are described (Pastrana et al. 2009; Platel et al. 2009; Platel & Bordey 2016; Fischer et al. 2011; Giachino et al. 2014) and in the dentate gyrus (Encinas et al. 2006; Encinas et al. 2011; Steiner et al. 2006; Pastrana et al. 2009; Platel et al. 2009; Platel & Bordey 2016; Lugert et al. 2010; Fischer et al. 2011; Giachino et al. 2014; Shors et al. 2014; Kempermann et al. 2015).

2. Glycosphingolipids

The primary function of the cell membrane is to offer barrier protection for the different components of the cells from the outside environment. Since the lifetime of the organic molecules such as proteins, nucleic acids, and lipids is much shorter than that of cells and, for example, the median half-life of a protein is only 46 hours (Schwanhausser et al. 2011; Schwanhausser et al. 2013), membranes exert an important role in shielding these high-turnover molecules.

The role lipid component of the membrane in the determination of the cell's attributes has recently reached a pivotal impact. The view of the cell membranes as a supporting structure that has the main role in containing proteins which are the depositary of controlling cell functions has changed.

The discovery of glycolipids (lipids covalently bound to carbohydrate) is usually attributed to Johan L. W. Thudichum which published is work of isolation of several compounds from brain extracts in the late 19th century (Thudichum 1884). Within the different compounds present in the extract he found one, which named phrenosin (nowadays termed galactosylceramide, (GalCer)), that produced three distinct products after acid hydrolysis, an isomer of glucose, a fatty acid and a novelle alkaloid compound with very enigmatic nature he named sphingosine (Sph) thinking about the Egyptian Sphinx.

The first structural characterization of Sph was performed in 1947 by Herb Carter as 2S,3R,4E-2-aminooctadec-4-ene-1,3-diol (Carter et al. 1947). In the following years, due to the set of methods for their analysis, more than 60 structural variations were detected (Karlsson 1970b; Karlsson 1970a). If the glycolipids contain one or more glycerol residues, they are termed glycoglycerolipids, whereas those containing either a ceramide (Cer) or a sphingoid are referred to as glycosphingolipids (GSLs) (Yanagisawa 2011). Cer is composed of a fatty acid and a Sph and it is the simplest sphingolipid (SL), it consists of a sphingoid base to which a fatty acid is attached at C-2 via N acylation and represents the backbone of all complex SLs. The bound of phosphorylcholine forms sphingomyelin (SM), and the bound of glucose or galactose represents the first step in the formation of GSLs.

In mammalian sphingoid bases are mostly 2S-amino-1,3R-octadecanediol (d18:0), 2-amino-4-octadecene-1,3-diol (d18:1), and (2S,3S,4R)-2-aminooctadecane-1,3,4-triol (t18:0). This is related to the preference of mammalian serine palmitoyltransferase (SPT) for saturated fatty acyl-CoAs with 16 ±1 carbon atoms, link with the abundance of palmitoyl- CoA (Merrill & Williams 1984); less significant amount of sphingoid with chain lengths between 12 to 26 was found (Pruett et al. 2008). In mammals brain the Cer lipid is mostly composed by a fatty acid ($C_{18:0}$, stearic acid) attached to one of two Sphs d 18:1 and 2-amino-4-dodecene-1,3-diol (d 20:1) (Jackson et al. 2011).

GSLs can be subdivided into neutral and acidic; the latter are referred to as gangliosides if they contain sialic acid residue(s) in their carbohydrate moiety (Yu et al. 2009).

Nowadays, the different components of the membrane, such as GSLs, not only have an important role in the maintenance of cell compartments but also exercise the duty of directing other membrane components (such as proteins) to the right places and they might also have a part in the modulation of protein's function. Moreover, GSLs have specific interactions with lectins or glycans on different cells and to the extracellular matrix. Some GSLs influence gene expression by interacting with chromatin and DNA regulatory elements; hence, they became an important target for pharmacological intervention.

SLs and GSLs are paramount structural components of mammalian cell membranes and they are mostly localized at the cell surface. SLs are constituted by Cer moiety with an N-acylated Sph group, usually galactose or glucose are bound to the primary

hydroxy group of the Sph forming the simplest GSLs: GalCer and glucosylceramide (GlcCer). The addition of further oligosaccharides generates a wide range of GSLs with variable complexity (Merrill et al. 2007). The important characteristic of GSLs is the heterogeneity of the molecular structures of their carbohydrate chains, due to a very entangled system of enzymes and the lipid and carbohydrate moiety.

The biosynthesis of SLs/GSLs is embedded in the cytosolic leaflet of the endoplasmic reticulum (ER) (Hannun & Obeid 2008). The first step is the condensation of a sphingoid base (either deriving from a *de novo* synthesis such as sphinganine, or a Sph in the recycle pathway) with an acyl-CoA, catalyzed by six enzymes: the ceramide synthases (CerS)1– 6 (Mullen et al. 2012).

The characteristics of the enzymes involved for each step were first described in the 1960s. Cer is the starting building element for complex SLs and GSLs and is formed by long-chain sphinganine or Sph, and a C2 amide-linked fatty acid (Karlsson 1970b). Transferases with different locations are responsible for the addition of sugars to Cer; for instance, the endoplasmic reticulum (ER) contains high levels of Cer galactosyltransferase (UGT8) that converts Cer into GalCer (Schulte & Stoffel 1993). Cer reaches Golgi with two alternative pathways; by the Cer transfer protein (CERT) from the ER to the cytosolic site of the trans-Golgi membrane, but rapidly achieve the equilibrium with the luminal side and it is mainly used for the synthesis of Sph (Hanada et al. 2003). Another pathway is most likely *via* vesicular transport (Gault et al. 2010) where Cer is modified to GlcCer) by Cer glucosyltransferase (UGCG) (Hanada et al. 2003), in both cases the synthesis happens in the cytosolic leaflet of early Golgi membranes. GlcCer can be transported in the lumenal membrane leaflet of the Golgi where can be glycosylated producing GSLs (van Meer et al. 2008). In an alternative pathway GlcCer is transported in the trans-Golgi network (TGN) and exposed in the lumenal membrane leaflet where can be further modified by glycosylation or sulfation (De Rosa et al. 2004), GlcCer and GalCer are the simplest precursors of more complex GSLs which are produced in the Golgi complex by different glycosyltransferases (Merrill 2011). The synthesis of more complex GSLs continues in the early Golgi (Trinchera et al. 1991), where a specific galactosyl transferase the β-1,4-galactosyltranferase (LacCer synthase, B4GALT6) produces lactosylceramide (LacCer). LacCer is the precursor of the most relevant GSL groups in mammals, their classification derives from their core carbohydrate

organization: ganglio-, isoganglio-, lacto-, neolacto-, lactoganglio-, globo-, isoglobo-, muco-, gala-, neogala-, mollu-, arthro-, schisto-, and spirometo-series (Yanagisawa 2011). More than 400 species, including neutral GSLs, sulfated GSLs, and gangliosides, have been identified in vertebrates (Sud et al. 2007; Yanagisawa 2011), having as building block Cer and more than 400 glycan structures that range from one to 20 sugar residues that can be further modified by sulfation. Galactose is the most frequent sugar featuring 40%, N-acetylglucosamine (GlcNAc) represents about 20%), glucose 14%, fucose 10%, N-acetylgalactosamine (GalNAc) 8%, N-acetylneuraminic acid 5%, and other sugar residues of more rare representation (D'Angelo et al. 2013). This wide number of product is achieved by means of different enzymatic activities: it is acted upon by α-galactosyltransferase (A4GALT) for the biosynthesis of globosides such as Gb3 (Galα1-4 Galβ1-4Glcβ1-Cer) (Kojima et al. 2000), by N-acetyl-glucosaminyltransferase B3GNT5 for the biosynthesis of lacto and neo-Lacto lipids (Biellmann et al. 2008), or by the enzymes devoted to ganglioside biosynthesis. In particular, N-acetyl-galactosaminyltransferase (B4GALNT1) is responsible for the synthesis of "o" series (asialo) gangliosides such as GA2 (Nagata et al. 1994), and lactosylceramide α-2,3-Sialyltransferase (ST3GAL5) for the others (a, b, and c) (Ishii et al. 1998). GSLs belonging to the globo/iso-globo, asialo, lacto/neo-lacto, and ganglio series originates from Gb3, GA2, Lc3, and GM3, respectively (Merrill 2011).

From the TGN GSLs are transported to the plasma membrane (PM) where can undergo to remodeling operated by specific glycosidases (Aureli et al. 2011), alternatively they are degraded entering in the lysosomes (Schulze & Sandhoff 2011).

2.1 *GSLs in the Central Nervous System*

The four major adult mammalian brain gangliosides, which account more than 97% of all gangliosides of the brain (Svennerholm 1964; Ngamukote et al. 2007), are GM1, GD1a, GD1b, and GT1b (Tettamanti et al. 1973) and they go through under remarkable changes in their relative composition during development (Kracun et al. 1991). These four gangliosides (Fig. 2) have a common neutral tetrasaccharide core (Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′Cer), with one or two sialic acids bound to the

internal galactose (GM1 and GD1b, respectively) and an additional sialic acid on the non-reducing terminal galactose (GD1a from GM1) and GT1b (from GD1b) (Fig. 2). The orientation of their molecule is of pivotal importance for their function. The Cer tail is embedded in the outer leaflet of the PM, with the GlcCer bond of ganglioside oriented such way that the glycan extends orthogonal to the surface of the lipid bilayer of the PM (Aureli et al. 2016).

Gangliosides have the main role in activity modulation of signaling proteins or receptor respectively acting in cis in their own membrane or in trans on the opposing cell (Regina Todeschini & Hakomori 2008).

Myelin-associated glycoprotein (MAG), which is produced by the oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system, is present selectively in the closest wrap of myelin with gangliosides on the opposing surface of the nerve cell axon (Schnaar 2010). Gangliosides GD1a and GT1b were found to contain motifs capable of bind MAG (Yang et al. 1996). The lack of GD1a and GT1b, which have the role of stabilizing the myelin, causes a dysmyelination (Schnaar 2016). The knockout of *B4galnt1* induces a loss of the complex ganglioside synthesis GD1a, GD1b, GM1, and GT1b and determines a concomitant production of quantitatively equivalent buildup of GD3 and GM3 (Takamiya et al. 1996). Mice with disrupted GM2/GD2 synthase gene lack complex gangliosides but exhibit only subtle defects in the structure and distributions at nodes of Ranvier (Susuki et al. 2007), axon degeneration due to dysmyelination (Sheikh et al. 1999; Furukawa et al. 2008), and, consequently, progressive motor neuropathy (Chiavegatto et al. 2000), indicating that gangliosides such as GD1a and GT1b are of pivotal importance axon-myelin stability.

In neuronal membranes, gangliosides are located on the external leaflet of the PM and represent the most abundant components (10–12% of the lipid content) (Posse de Chaves & Sipione 2010).

In the CNS only one glycoglycerolipid has been found, the phosphatidylglucoside (PtdGlc) containing a single glycerol (Nagatsuka et al. 2006). By immunostaining in mice CNS it was found a stage- and cell type-specific localization of the PtdGlc antigen during development, with high expression levels in radial glia/astroglia (Nagatsuka et al. 2006). In addition, PtdGlc was also detected NSCs/neural precursor cells in the SVZ, and since then it was considered a marker of adult NSCs (Kaneko et al. 2011).

Interestingly, the glucosyl cholesterol, which was firstly found in plants (Grille et al. 2010) and lately in mammalian cells (Kunimoto et al. 2000), has been also found in brain (Marques et al. 2016). This metabolite might have a role as an intermediate in a transport pathway since is more soluble than cholesterol.

2.2 Membrane domains in brain cells

In addition to its shielding role, the membrane also participates in important cellular functions such as signaling and cell-to-cell communication that are evident in nerve cells, having a highly polarized PM that plays a variety of important roles in signaling.

In this context, membrane functions are highly influenced by membrane morphology and molecular composition, which allows us to hypothesize that the membrane may control in various ways the neuronal signaling.

This increasing number of functions of the cell membranes could not be explained by the original theory of the homogeneous fluid lipid bilayer, as described by Singer and Nicholson in the early seventies (Singer & Nicolson 1972). Indeed, it has become clear, as mentioned earlier, that the PM is composed of thousands of different lipids, this complexity underlies a much more intricate structure than previously believed.

The complexity of the cellular membrane is clearly evident in nerve cells. A high degree of diversification is due to the enormous number of different cells, but there are many different levels of intricacy within each type of cells. Firstly, interneurons, motoneurons, astrocytes, oligodendrocytes and others all have different characteristics due to their function. Second, different regions of the nerve cells, important for disparate cell functions, have different cell lipids composition.

As a matter of fact, the relationship between the organization of particular membrane sub-compartments, at a variety of scales, and the cellular and tissue activity, is more by far obvious than in any other tissue. The majority of nerve cells (including glia) are extremely polarized with various morphologically identifiable and well-typified membrane “macrodomains”; for instance, the multi-layered myelin sheath in oligodendrocytes and the synaptic membrane macrodomains in neurons (Poliak & Peles 2003).

On the other side, membrane regions that lack a morphologically recognizable architecture have membrane elements that do not move in free, sustained lateral diffusion but are restricted to lipid domains called rafts (“micro-” or “nanodomains”)

(Jacobson et al. 1995) which are present in both the layers of the membrane (Rietveld & Simons 1998).

Stimulated Emission Depletion (STED) microscopy has shown that the confinement of glycosylphosphatidylinositol (GPI)-anchored proteins is enclosed in conjunction with membrane components such as monosialoganglioside GM1 (that was first identified as detergent-insoluble material, DIM), a further analysis indicated that DIM was also composed by other SLs such as GM2, gangliosides, cholesterol and SM, lipid-anchored proteins (containing GPI anchors or fatty acid modifications) (Sonnino & Prinetti 2013) and other PM proteins of hydrophobic nature such as caveolin (Sargiacomo et al. 1993) with a tiny membrane areas with diameter >20 nm, and a lifespan of 10–20 ms (Eggeling et al. 2009; Eggeling 2015). As an example, the post-synaptic density present in neurons is highly organized, with an impressive variety of neurotransmitter receptors at the post-synaptic site. This organization depends on a complex varied set of lipid scaffolding and protein adaptors that modulate the effectiveness of synaptic transmission (Hemler 2005; Lindner & Naim 2009). The initial theory in which the lipid rafts were described as exclusive lipid composition was afterward modified introducing the role of structural proteins such as the actin meshwork, located under the membrane, in regulating membrane-protein clustering, lateral diffusion, and raft stability (Kusumi et al. 2005).

PM and endosomes of eukaryotes contain elevated levels of SLs and sterols. SLs usually contain long saturated fatty acids (C16–C32) with an amide linkage to the sphingoid base; they assume a solid gel phase, but sterols make them more fluid. Other studies have indicated a very high proportion of phospholipids and cholesterol in the cytosolic leaflet (Mondal et al. 2009).

Due to increased cell complexity and the need for more complicated interactions between cells, cells began, during evolution, to employ particular lipids at a particular position for signaling. Many GSL structures on the cell surface (Li et al. 2010) specifically interact with glycans or lectins on near cells or to the extracellular matrix. They can also balance the activation of the insulin growth factor receptor (IGFR) and epidermal growth factor receptor (EGFR) (Bremer et al. 1986) and of integrins by changing their structure becoming lipid rafts (Todeschini et al. 2008). Another level of complexity is seen in these lipid rafts, due to the self-associative attributes of the apical lipids (cholesterol and GSLs), controlling the production of liquid-ordered (*lo*) phase domains “lipid rafts” (Ipsen et al. 1987). Twenty-one years

after Ipsen et al demonstrated that SLs and sterols are separated from glycerolipids in the trans-Golgi system throughout trafficking (Klemm et al. 2009). It was subsequently suggested that the spontaneous partition of defined membrane lipids, responsible for the formation of *lo* phase domains are a common sorting and targeting process of membrane constituents (Simons & Ikonen 1997).

With the discovery of the complexity of lipids membrane, a less *protein-centric* vision, in which protein-protein interactions were considered the most important feature, was proposed (Marth 2008), accounting for the ballast of membrane macro- and micro-domains. The role of membrane lipids in regulating the lateral management of the membrane has emerged in the last 15 years.

The amino acid sequence termed the "sphingolipid binding domain" is present in many proteins that interact with GSLs, some of which, the amyloidogenic proteins such as α-synuclein and β-amyloid peptide, contain a loop located around a tyrosine amino acid, that is necessary for their capacity to bind GSLs (Fantini & Yahi 2011). Moreover, many receptors including the human serotonin1A receptor (Prasanna et al. 2016), the human β2-adrenergic receptor (Hanson et al. 2008), the nicotinic acetylcholine receptor (Corbin et al. 1998) and the γ-aminobutyric acid (GABA) and glutamate receptors (Becher et al. 2001) are associated with lipid rafts. In some cases, even if a specific lipid-binding motif is missing in a protein, a "shell" of typical raft lipids could be present, allowing a high degree of interaction between proteins and lipids (Fantini & Barrantes 2009).

Given the above, the interaction of a protein with a *lo* phase with smaller fluidity than the neighboring bilayer may be a way of reducing the lateral movement of the protein and support further stable interactions with other proteins located in the same domains. Moreover, the interaction of a protein with a stiff membrane surface might influence conformational alterations in the polypeptide chain modifying its functional activity. Finally, protein-lipid raft interaction is favored by the elevated protein concentration (Levade & Jaffrezou 1999).

3. GSLs in Neural Stem Cells

Gangliosides have important roles in the development of the nervous system. The signaling involved in NSCs fate regulation, mediated or modulated by glycoconjugates, is referred to as "glycosignaling". Diverse gangliosides have been

described as being expressed during development (for a schematic description of the distribution and role of GSLs see Table 2).

During the development of the brain, gangliosides seem to modulate Cer induced apoptosis (Bieberich et al. 2001). Specifically, transgenic mice lacking neutral glucosphingolipids (glucosyltransferase knockout) or complex gangliosides (B4GALNT1 knockout) shown a high incidence of apoptosis in the nervous system during embryonal development or adulthood (Sheikh et al. 1999), with spatial and temporal effects for the selective regulation (Bieberich et al. 2001).

For instance, GD2 is present in human embryonic and mouse postnatal NSCs (Klassen et al. 2001), whereas GD3, GT1b, and GQ1b can be found in mouse embryonic neural precursor cells (NPCs) (Yanagisawa et al. 2004). It has been proposed that these b-series gangliosides (Yu et al. 2004) are important markers of NSCs/NPCs.

Indeed, during development, GM3 and GD3 are highly expressed during neural tube formation in neuroepithelial cells (NEC), in radial glial cells (RGCs) of the embryo ventricular zone, and NSCs proliferation of the adult SVZ; they then decrease in neural differentiation. GM1, GD1a, GD1b, and GT1b increase during neural differentiation and are elevated during axonal/ dendritic arborization, synaptogenesis, myelination, up to adulthood (Olsen & Faergeman 2017) (Table 2).

Ganglioside GD3 is considered as a marker of immature neuroectodermal cells for quite some time (Nakatani et al. 2010). GD3 is the main species in embryonic (E14) mouse striatum and early postnatal (P2-10 SVZ) (80%), whereas they decrease up to 30% in adult (P30 (SVZ) as shown by means of immunological staining using the monoclonal antibody R24 (Nakatani et al. 2010; Wang & Yu 2013). A decreased self-renewal capability was observed in NSCs isolated from GD3-synthase (ST8SIA1) (GD3S)-KO mice related to those obtained from wild-type animals (Table 2). This reduction correlated with a reduced expression of EGFR, which colocalized with GD3, and a larger degradation rate of EGFR and EGF. These phenomena were accompanied by a switch, in the GD3S-KO NSCs, of EGFR that was found mainly in the high-density layers (Wang & Yu 2013). It was found that GD3 is necessary for the long-term preservation of NSC populations in the postnatal mouse brain (Wang et al. 2014) and that GM1 and GD3 at low concentrations (from 0.001 to 10 μM) are important factors for NSCs proliferation whereas higher concentrations (50 and 200 μM) induced a reduction in proliferation (Itokazu et al.

2013; Wang et al. 2016). EGFR interaction with the N-linked GlcNAc oligosaccharides portion of GM3 (NeuAcα2-3Galβ1-4Glcβ1-1′Cer), in a cis fashion (Regina Todeschini & Hakomori 2008), which has a negative impact on the EGFR tyrosine kinase (Hanai et al. 1988), was first reported in Hakomori's laboratory. Moreover, its NeuAc-lactose core was demonstrated to be of pivotal importance for its interaction with EGFR extracellular domains (Miljan et al. 2002). Since GD3 arises from GM3 adding an α2–8-linked sialic acid to its sialic acid residue, it has in common analogous structural characteristics with GM3, making it probable that the carbohydrate elements could be accountable for the interaction between EGFR and GD3. Indeed, it is already known that the extracellular domain (ECD) of the EGFR binds directly to ganglioside GM3 (Miljan et al. 2002) and modulates kinase activity. The relative binding capability *vis-à-vis* GM3 of other gangliosides can be describes as follows: GM3 (100%)> GD3 (49%)> GM4 (43.5%)> GM2 (29%)> GT1b (17.2%)> GM1 (12.1%)> GD1a> lactosylceramide (11.6%), indicating that NeuAc-lactose is crucial for binding and that any sugar substitution decreases binding (Miljan et al. 2002). Interestingly, GD3 is a most important glycolipid component of the immature vertebrate CNS, but an unimportant factor within the mature CNS. Using antibodies against GD3, it was found that in postnatal rat CNS there are several areas enriched by this epitope such as the SVZ and the dentate gyrus of the hippocampus (Goldman et al. 1984) (Table 2).

It is believed that lipid rafts have high affinity and activation capacity to specific cell signaling proteins in particular cytokine receptors and growth factors, which leads to clustering of these receptors in different types of cells. Hence, we cannot exclude the possibility that these lipids can influence NSCs neurogenesis with at least two different mechanisms: direct interaction with lipid receptors such as G protein-coupled receptors (GPCRs) and lipid raft-dependent activation of growth factor or cytokine receptors. Beyond EGFR, other candidates are leukemia inhibitory factor receptor α (LIFR α), IGFR, or fibroblast growth factor receptor 2 (FGFR2) each of which has an important role in the proliferation and differentiation of NSCs.

In murine NSCs, GD3 expression coexists with that of stage-specific embryonic antigen (SSEA) 1, another murine NSCs marker, in neurospheres but not in differentiated cells. For this reason, GD3 can be believed to be a useful NSCs surface marker (Nakatani et al. 2010). Moreover, the ganglioside 9-O-acetyl GD3, a glycolipid on type A cells, is required for cells migration. As expected, blocking this

ganglioside by the use of Jones monoclonal antibody (Jones mAb) causes neuroblast migration in a dose-dependent manner (Miyakoshi et al. 2001) (Table 2).

As already mentioned a shift in GSLs is common during neurogenesis *in vitro* (and *in vivo*). In particular, if the levels of GD3 remain elevated during cell division the cells maintain their stemness; in contrast, if the levels of GD3 reduce a more mature progeny is generated; these changes are related to an increase in histone acetylation, which leads to the developmental changes of ganglioside expression. The interaction between histones and DNA responsible for the relaxation of chromatin can be altered by the acetylation of histone H3 or H4, resulting in transcriptional activation and repression (Hsieh & Gage 2004). In NSCs this phenomenon boosts neuronal differentiation and impedes glial differentiation by means of the regulation of neurogenic transcription factors (Hsieh et al. 2004). Recently it was suggested that glycans could play a part as modulators in epigenetic gene regulation. Glycosyl modification such as OGlcNAcylation was demonstrated to regulate the localization, activity, and stability of many transcription factors, controlling their interactions with DNA and other proteins, suggesting the important role of this post-translation modification for the epigenetic control of gene expression (Lewis & Hanover 2014). The efficient histone acetylation of glycosyltransferase genes in mouse brain has an important role for the developmental alteration of ganglioside expression (Suzuki et al. 2011) *via* trans-activation factors such as SP1 and AP-2 (Tsai & Yu 2014). While GD3 interacts with histone H1 in the nucleus (Tempera et al. 2008), GM1 is located at the nuclear periphery and colocalized with lamin B1 and nucleoporin of neuronal cells derived from NSCs, indicating that chromatins occupied by GM1 are transcriptionally active (Itokazu et al. 2017). Indeed, the addition of GM1 in NSCs culture led to neuronal differentiation, indicating the role of this molecule in the fate of the NSCs and a sort of positive loop that led to an increase in the level of GM1 and other complex forms of brain-type-gangliosides, such as GD1a, GD1b and GT1b by raising the GalNAcT message level (Itokazu et al. 2017).

GM1 may be a pivotal player in deciding axonal fate; for instance, Neu3 (Sialidase-3), which converts more complicated gangliosides to GM1, is crucial for establishing axon specification and regulating apoptosis. Others Sialidases such as Neu4 are associated to PM exerting function in the mitochondria (long form) (Yamaguchi et al. 2005) or intracellular membrane (short form) (Monti et al. 2004). Ganglioside sialidase (PMGS) induces an actin instability by asymmetrically accumulating at the

tip of one neurite. PMGS can selectively hydrolyze gangliosides producing PM rich in GM1. Elevated PMGS activity induces axon specification by increasing tropomyosin receptor kinase A (TrkA) activity locally, which prompts phosphatidylinositol- 3-kinase (PI3K)- and Rac1-dependent inhibition of RhoA signaling and the concomitant actin depolymerization in only one neurite (Da Silva et al. 2005).

C-series GSLs are mostly expressed in glia lineage. The antigens recognized by the A2B5 antibody are present in GQ1c, Gt1c, and GT3 (Saito et al. 2001). Although less represented in the adult mammalian brain (Rosner et al. 1988), this series can be recognized in glia restricted precursors that later produce type 1 and 2 astrocytes and oligodendrocytes.

4. Conclusions

Recent research encrypted the role of GSLs in cell fate.

Adult NSCs have been considered a reservoir of cells with few (known) roles in the human brain, but with extensive impact in lower mammals. The cultivation, expansion, and differentiation of NSCs *in vitro* is an important aspect that will have to be taken into account if we are to consider using those cells as pharmacological tools for the treatment of neurological diseases; it is therefore crucially important to understand the molecular determinants that have a role in neurogenesis *in vivo* and *in vitro*.

The dose-dependent effects of GM1 and GD3, for instance, are most intriguing (Itokazu et al. 2013; Wang et al. 2016). It is already known that NSCs express GD3 as the most abundant ganglioside (Nakatani et al. 2010), and co-localize with EGFR regulating cell proliferation (Itokazu et al. 2013) (Fig. 3); moreover, GD3 (at high concentration) is involved in mitochondria permeability transition preceding apoptosis (Scorrano et al. 1999) interacting with β-amyloid (Fig. 3). Therefore the effect of this ganglioside could depend on its concentration and the culture condition (i.e. the presence of β-amyloid which it is known to induce (β-amyloid 25-35) elevation of intracellular levels of GD3 (Copani et al. 2002)). Interestingly some gangliosides (GM1, GD1a, GD1b, and GT1b) induced an inhibition of EGFR tyrosine phosphorylation and EGF-stimulated cell proliferation neuroblastoma tumor cells of human origin (Fig. 3) (which contain cancer stem cells of neural origin) (Mirkin et al. 2002).

It is also interesting the role of glycosphingolipid complexity in NSC differentiation. The correct level of complexity is important for the TrkA signaling response changing the cellular localization of nerve growth factor receptors (NGFR) (Olsen & Faergeman 2017) (Fig. 3). Moreover, GT1b has a structural function in axonal membrane microdomains, playing a part in myelin stability by interacting with MAG, and this has a further role in the differentiation and neuron maturation of NSCs (Olsen & Faergeman 2017).

Individual GSLs do not have a unique role in NSC differentiation. For instance, one can induce self-renewal in a stem cells population, but exert an induction of differentiation in another type; conversely, the same type of cells from different organisms can be regulated in opposite fashion by different GSLs.

Up to date, many bioactive lipids interface with receptors located in cell, nucleus, and endoplasmic reticulum membrane. Sphingosine-1-phosphate (S1P) acts through membrane receptors activating GPCRs of the inhibitory (Gi), phospholipase (Gq), and regulatory activity of the actin cytoskeletal remodeling in cells (G12/13) type. These GPCRS (triggered by lipids) are some of the cell signaling pathways (Phosphoinositide 3-kinase (PI3K)-to-Akt (Gi), Phospholipase C (PLC)-to- Protein kinase C (PKC) (G12/13), and Ras-to-Extracellular signal–regulated kinase (ERK) (Gi, Gq)) for NSC proliferation and differentiation. Often the characteristic cellular response (e.g., differentiation vs proliferation) is arranged by a peculiar association of bioactive lipids.

Two main regulators of ES neural differentiation are protein phosphatase 1 (PP1) and 2a (PP2a), which are involved in the de-phosphorylation of many protein kinases or other proteins included, but not limited, to an atypical (a)PKC upstream of Akt and Glycogen synthase kinase-3β (GSK-3β) (Wang et al. 2005; Krishnamurthy et al. 2007). The most important functions of aPKC for embryo development and stem cell differentiation are cell- and tissue-specific; for instance, the inhibition all isoforms of aPKC preserves self-renewal and hampers lineage commitment of ES cells (Das et al. 2011). A few years ago, Bieberich's group determined the presence of a binding site for Cer in the C-terminus of aPKC different from the domain in the regulatory moiety of the enzyme (Wang et al. 2009). The bound of Cer to aPKC may take place in a homologous fashion as binding happens between Diacylglycerol (DAG) and PKCα, for instance, the C2 domain binds to Ca^{2+} /PIP2 or phosphatidylserine (PS) and stimulates the binding of the C1a domain to DAG (Cho & Stahelin 2005). The

author also hypothesized that the binding of Cer to aPKC is associated with PIP_2 or PIP_3 Cdc42 binding, which interacts with by Par6 to the N-terminus of aPKC (Bieberich 2008). Moreover, the binding of Cer-aPKC to either PAR-4 or Par6-Cdc42, which are mutually exclusive, may account for a "change in cell fate" and may be responsible for the control of apoptosis (PAR-4) and cell polarity (Par6-Cdc42) balance in NSCs (Bieberich 2008; Bieberich 2011). Cer affects NSC differentiation by means of two mechanisms: the equilibrium between Cer and its derivative S1P and that between Cer and other GSLs. Cer maintains cell polarity and nurture differentiation, meanwhile, S1P enhances cell survival.

Within various GSLs, GM1 is able to inhibit PKC movement from cytosol to membrane (Cabedo et al. 1996). In murine neuroblastoma (Neuro-2a model/line), GD1 and BIM (a PKC inhibitor) are able to induce neuritogenesis (Singleton et al. 2000), in a synergistic fashion, indicating that most likely GD1 acts on NSCs differentiation by means of altering the cellular localization of the PKC.

GD1 seems to modulate neuritogenesis by the regulation of Ca^{2+} influx to the nucleus of Neuro-2A cells (Wu et al. 1990); moreover, in undifferentiated NG108-15 cells, GD1 is able to modulate Na–Ca exchanger in the nuclear envelope (Xie et al. 2002). Taken together this information indicate that the effectiveness of GSLs may not be sufficient for the activation of differentiative or proliferative events themselves, but rather they could act via the enhancement of the activity of other factors.

To date, the regulation of NSCs *in vitro* has been performed with factors of protein origin, but lately, the role of GSLs in neurogenesis was considered to have a focal impact. The GSLs might have an unpredictable impact on our capacity to regulate NSCs and might play, in the near future, an important role in the control of neurodegenerative pathologies.

Abbreviations

Neural stem cells (NSCs); central nervous system (CNS); sub ventricular zone of the lateral ventricle (SVZ); subgranular zone (SGZ); intermediate progenitor cells (IPCs), neural/glial antigen 2 (NG2) progenitors; glial fibrillary acidic protein (GFAP); calcium-binding protein B (S100B); glycosphingolipids (GSLs); sphingosine (Sph); ceramide (Cer); Sphingolipids (SLs); endoplasmic reticulum (ER); glucosylceramide (GlcCer); Cer transfer protein (CERT); Cer glucosyltransferase (UGCG); trans-Golgi network (TGN); galactosyl transferase the

β-1,4-galactosyltranferase (LacCer synthase, B4GALT6); N-acetylglucosamine (GlcNAc); N-acetylgalactosamine (GalNAc); N-acetylneuraminic acid (NeuAc); lactosylceramide (LacCer); α-galactosyltransferase (A4GALT); Gb3 (Galα1-4 Galβ1-4Glcβ1-Cer); N-acetyl-galactosaminyltransferase (B4GALNT1); lactosylceramide α-2,3-Sialyltransferase (ST3GAL5); plasma membrane (PM); myelin-associated glycoprotein (MAG); phosphatidylglucoside (PtdGlc); Stimulated Emission Depletion (STED); glycosylphosphatidylinositol (GPI); sphingomyelin (SM); insulin growth factor receptor (IGFR); epidermal growth factor receptor (EGFR); γ-aminobutyric acid (GABA); neuroepithelial cells (NEC); radial glial cells (RGCs); GD3-synthase (ST8SIA1); extracellular domain (ECD); G protein-coupled receptors (GPCRs); leukemia inhibitory factor receptor α (LIFRα); fibroblast growth factor receptor 2 (FGFR2); stage-specific embryonic antigen (SSEA) 1; Neu3 (Sialidase-3); Neu4 (Sialidase-4); ganglioside sialidase (PMGS); tropomyosin receptor kinase A (TrkA); phosphatidylinositol- 3-kinase (PI3K); nerve growth factor receptors (NGFR). S100 calcium-binding protein B (S100B); Signal transducer CD24 (CD24); Gap junction alpha-1 protein (GJA1), also known as connexin 43 (Cx43); prominin-1 (CD133); Glial fibrillary acidic protein (GFAP), Nestin is a type VI intermediate filament (IF) protein, Brain lipid-binding protein (BLBP); Epidermal growth factor receptor (EGFR); Calretinin is a calcium-binding protein; Neuronal Nuclear Antigen (NeuN); Doublecortin (DCX); Polysialylated-neural cell adhesion molecule (PSA-NCAM); Neurogenic differentiation 1 (NeuroD); Prospero homeobox 1 (Prox1); Calbindin calcium-binding protein; Achaete-scute homolog-1 (termed Mash1 in rodents, hASH1 in humans); Lewis X (Lex); Distal-less homeobox 2 (Dlx2) ; Neuron-specific class III beta-tubulin (Tuj1); Sex determining region Y)-box 2 (Sox2); Sphingosine-1-phosphate (S1P); Phosphoinositide 3-kinase (PI3K); Phospholipase C (PLC); Protein kinase C (PKC);Extracellular signal–regulated kinase (ERK); Protein Phosphatase 1 (PP1); Protein Phosphatase 2a (PP2a); Glycogen synthase kinase-3β (GSK-3β); Diacylglycerol (DAG).

Figure legend

Fig. 1

Schematic representation of the mouse central nervous system.

A: sagittal section of the mouse brain including the spinal cord;

B: coronal section of the mouse brain at the level of the sub-ventricular zone (SVZ in green);

C: coronal section of the mouse brain at the level of the dentate gyrus (DG in red);

D: coronal section of the mouse brain at the level of the spinal cord.

3^{rd} V third ventricle, 4^{th} V third ventricle, LV: lateral ventricle, CC central canal of the spinal cord (in blue).

Fig. 2

Structure of the four most representative GSLs of the mammalian brain: GM1, GD1a, GD1b, and GT1b.

Fig. 3

Hypothetical representation of the tyrosine kinase interaction with GSLs involved in NSCs physiology, in red there is indicated the activating pathways in blue the inhibitory pathways.

Table legend

Table 1

Schematic description of the cell markers present in NSCs from different mouse brain regions. S100 calcium-binding protein B (S100B); Signal transducer CD24 **(**CD24); Gap junction alpha-1 protein (GJA1), also known as connexin 43 (Cx43); prominin-1 (CD133); Glial fibrillary acidic protein (GFAP), Nestin is a type VI intermediate filament (IF) protein, Brain lipid-binding protein (BLBP); Epidermal growth factor receptor (EGFR); Calretinin is a calcium-binding protein; Neuronal Nuclear Antigen (NeuN); Doublecortin (DCX); Polysialylated-neural cell adhesion molecule (PSA-NCAM); Neurogenic differentiation 1 (NeuroD); Prospero homeobox 1 (Prox1); Calbindin calcium-binding protein; Achaete-scute homolog-1 (termed Mash1 in rodents, hASH1 in humans); Lewis X (Lex); Distal-less homeobox 2 (Dlx2) ; Neuron-specific class III beta-tubulin (Tuj1); Sex determining region Y)-box 2 (Sox2).

Table 2

Schematic description of the distribution and crosstalk of GSLs in NSCs and NPCs.

Conflicts of interest

The authors declare that this research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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Table 1

Region	Cell Type	Markers	References
SVZ	**E**pendymal cells	S100B, CD24, Cx43, CD133	Platel et al. 2009
SVZ	**Q**uiescent B1 cells	GFAP	Platel et al. 2009
SVZ	**A**ctivated B1 cells	GFAP, Nestin, BLBP, CD133, EGFR	Platel et al. 2009, Giachino et al.2014, Fischer et al. 2011.
SVZ	B2 cells	GFAP, Nestin Cx43, BLBP	Giachino et al. 2014
SVZ	Astrocytes	GFAP, S100B	Platel et al. 2009
SVZ	C cells	Mash1, EGFR, Lex, Dlx2, BLBP	Pastrana et al. 2009, Giachino et al. 2014
SVZ	A cells	DCX, Tuj1 PSA-NCAM	Pastrana et al. 2009), Platel & Bordey 2016.
SGZ	RGL type 1	GFAP, BLBP, Nestin, and Sox2	Shors et al. 2002, Kempermann et al. 2015
SGZ	Transiently amplifying progenitor cells type 2a	$GFAP^{+/-}$, Nestin, BLBP and Sox2	Shors et al. 2002, Encinas et al. 2006, Kempermann et al. 2015
SGZ	Transiently amplifying progenitor cells type 2b	Nestin, DCX, PSA-NCAM, NeuroD, Prox1	Steiner et al. 2006
SGZ	Neuroblasts type 3 cells	DCX, PSA-NCAM, NeuroD and Prox1	Kempermann et al. 2015, Encinas et al. 2006
SGZ	DG neurons	Calretinin, NeuN, DCX, PSA-NCAM, NeuroD, Prox1 and Calbindin.	Lugert et al. 2010, Kempermann et al. 2015

Table 1

Table 2

Table 2

Glycosphingolipids	Cell line/tissue	Crosstalk/effect	Reference
GD2	Human embryonic and mouse postnatal NSCs	Highly expressed	Klassen et al 2001
GD3, GT1b, GQ1B	Mouse embryonic NPCs	Highly expressed; possible NSCs and NPCs markers	Yanagisawa et al. 2004; Yu et al. 2004
GM3, GD3	Neural tube and NSCs	Highly expressed during neural tube formation and NSCs proliferation, low expression in neural differentiation	Yanagisawa et al. 2004
GM1, GD1a, GD1b, GT1b	Adult nervous system	Increase during neural differentiation and elevated during axonal/ dendritic arborization, synaptogenesis, myelination, up to adulthood	Olsen and Faergeman 2017
GD3	Neuroectodermal cells, in SVZ	Considered as a marker, expressed in developing mouse brain and in NSCs	Nakatani et al. 2010; Wang & Yu 2013
GD3	GD3 synthase -KO in NSCs	GD3 interacts with EGF receptor system. KO of GD3, which colocalizes with EGFR, induces a degradation of EGF and EGFR. EGFR switch (presence of high density layers). It determines impaired neurogenesis at the granular cell layer of the olfactory bulb and the DG in the adult	Wang & Yu 2013
GD3	Postnatal mouse brain NSCs	Long term preservation of NSCs populations in postnatal mouse brain	Wang et al. 2014
GD3	NSCs culture	Interacts with histone H1 in the nucleus	Tempera et al. 2008
GM1, GD3	Postnatal mouse brain	At low concentration they induced proliferation; at high concentration they caused a reduction of proliferation	Itokazu et al. 2013; Wang et al. 2016

Table 2

GM1	NSCs culture	Colocalized with lamin B1 and nucleoporin indicating that chromatins occupied by GM1 are transcriptionally active	Itokazu et al. 2017
GM1	Axon specification	Ganglioside sialidase induces an actin instability by asymmetrically accumulates at the tip of one neurite, and increasing the levels of GM1. PMGS elevate activity induces axon specification by enhancing TrkA activity	Da Silva et al. 2005
NeuAc-lactose portion of GD3	Postnatal rat CNS: SVZ and dentate gyrus	Important determinant for interaction with EGFR for modulating its kinase activity	Miljan et al. 2002; Goldman et al. 1984
9-O- acetyl-GD3	Express in the rostral migratory stream *in vivo* (in type A cells) as well as in chains of neuroblasts that migrate from SVZ explants *in vitro*	Its inhibition causes the abolition of neuroblast migration in a dose-dependent manner	Miyakoshi et al. 2001
GQ1c, Gt1c, GT3	Present in glia restricted precursors that produce type 1 and 2 astrocytes and oligodendrocytes	These antigens are recognised by the A2B5 antibody	Saito et al. 2001