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MOLECULAR MECHANISMS INVOLVED IN KRABBE'S DISEASE

THESIS

Tutor: Prof. Venera Cardile

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To my parents, Antonina and Giovanni Graziano,
my sister, Maria Cristina, my nephew, Giulia,
for their never-ending love, encouragement and support.
To my extraordinary Tutor Prof. Venera Cardile
for revealing to me the endless appeal of
Neurosciences and Cell Physiology

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PREFACE

Usually, when common peoples talk about nervous system the first thought is directly addressed to brain and neurons, forgetting that glial cells constitute the large majority of cells in the nervous system (Pfrieger and Barres, 1995). Moreover, for long time neuroscientist attention was often concentrated almost exclusively on impulses and synapses.

The "neuronal doctrine" penalized and maintained in shadows oligodendrocytes and astrocytes by considering neurons and synaptic contacts as the only centre of nervous system and by assigning to glia a simple supporting role (Verkhratsky, 2007).

Since its identification, glia was described as "cells other than neurons" by Rudolph Virchow who called them "nervenkitt" (nerve glue), i.e., "neuroglia" (Virchow, 1846), considering them as the connective tissue of the brain.

Today, even if the name survives, the original concept radically is changed and the glia role during development, the active participation in the physiology of the brain and the consequences of their dysfunction on the pathology of the nervous system are objects of interest and emphasis.
It is clear glial cells are necessary for correct neuronal development and the functions of mature neurons. Their ability to respond to changes in the cellular and extracellular environment is essential to the function of the nervous system. Furthermore, there is now growing recognition that glia, possibly through a glial network, may have communication skills that complement those of the neurons themselves. For example, it seems likely that oligodendrocytes have functions other than those related to myelin formation and maintenance, but the number of studies on it appears like a small light in a very dark night. It can be quickly demonstrated by using each glial cell type as a keyword in Pubmed (Fig.1).

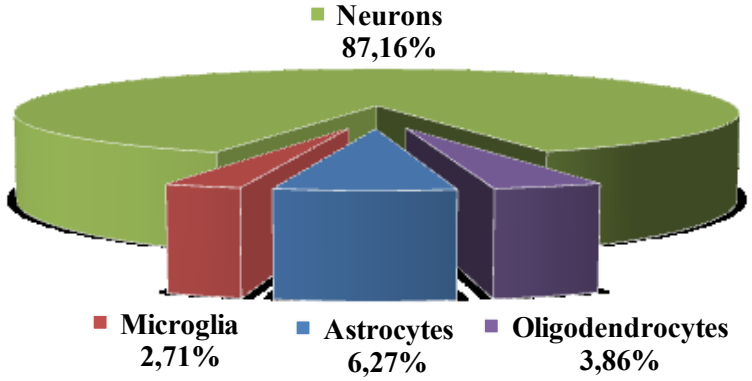

Fig. 1. Percentage of numbers of items reported on Pubmed

In 2013, more of 17,000 published articles focus on neuron; a large number despite the 800 works about oligodendrocytes in the same year.

The lack of research and the absence of a consistent background in literature about oligodendrocytes role and response in physiological conditions make more difficult the understanding of their behavior in pathology, and it opens new unexplored field.

A number of typical oligodendrocytes disorder represents the major challenge, like the dysmyelinating and demyelinating genetic diseases that involve genes in critical pathways for myelin formation, such as for defects in lipid metabolism. Among them Krabbe disease has been recently emphasized, as shown by Pubmed search (Fig. 2), but, still today, the involved molecular mechanisms are not fully investigated.

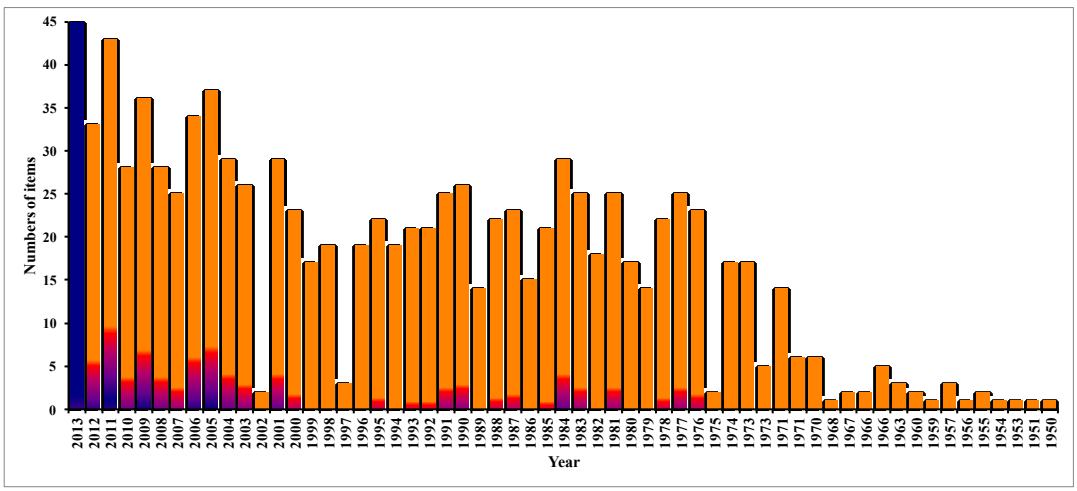

Fig. 2. Published articles regarding Krabbe disease
Since 1950, the total number of investigations is 1059
[Adapted from PubMed]

The principal cause of this situation could be due to poor interest in investing time and money for a rare pathology, even if extremely disabling and lethal.

Designed and developed in the described background, this study is a contribution to the knowledge of Krabbe disease. It was inspired and motivated by the history of Grazia, a little angel.

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"Stay Hungry. Stay Foolish!"

The Whole Earth Catalog, 1970
Stewart Brand

ABSTRACT

Krabbe disease is an autosomal recessive disorder resulting in the deficiency of galactosilcerebrosidase (GALC), a lysosomal enzyme involved in the catabolism of two lipids extremely represented during formation of myelin sheet: galactosylceramide and psychosine. Despite the GALC activity deficiency, galactosylceramide does not increase in the brain of patients, while psychosine accumulation is reported as cause of myelin-generating cells death and of severe neurodegeneration. The present work outlined a molecular explanation for how psychosine mediates a dose-dependent cell death in oligodendrocytes precursor and fibroblast cells with and without GALC mutation. In addition, it was analyzed how this mechanism may interfere in oligodendrocytes maturation. The connexins play essential roles in cell homeostasis, growth, differentiation and death. Among connexin family members, Connexin43 is recently reported as an active element in apoptosis by gap junction-dependent or -independent mechanisms. Still today, there are no scientific researches evaluating Connexin43 expression and localization in normal and in GALC mutated oligodendrocytes precursor cells. The results of this study established that the different effects of exogenous psychosine addition could be linked to different sensitivity of cell membrane. Furthermore, it was determined a primary involvement of cell proliferation and a relationship between caspase-3 activation and disturbance of membrane-activated protein involved in cellular growth and survival. Psychosine treatments resulted in proteolysis of procaspase-3 proportioned to PTEN up-expression and PI3K activity down-regulation, confirmed by Bad release, p53 enhanced levels and decrease of NF-kB. The intrinsic apoptosis activation was established. Moreover, psychosine–treated oligodendrocyte precursors increased Connexin43, demonstrating preferential intracytoplasmatic localization and indicating that no gap- junction was involved. These *in vitro* findings were supported by examination of Connexin 43 levels in mouse brains: wild-type mouse brain proteins were compared to those ones of twitcher mouse, a natural occurring model of Krabbe disease. Reminding that Connexin43 never was found in mature oligodendrocytes, the second part of this work was pioneer in evaluating Connexin43 expression during wild-type- and GALC-mutated oligodendrocytes maturation steps without psychosine treatment. In experimental condition applied, it was verified that wild-type oligodendrocyte progenitors differentiate into a mature phenotype with loss of Connexin43. Under the same condition, GALC-mutated oligodendrocyte precursor did not express marker of maturity, they became multinuclear and Connexin43 levels remained higher than control, suggesting that psychosine is accumulating in early steps of oligodendrocyte precursor cell maturation. Taken together, these findings led to the general idea that psychosine may functionally impact cells through interference on the growth system and alteration in maturation programme, strongly related to Connexin 43 expression. Thus, the third part of this work probed the likelihood of using adipose tissue-derived mesenchymal stem cells (AT-MSCs) as source of oligodendrocytes. Two different conditioned media were

used and the results demonstrated that AT-MSCs can be influenced by the environment versus a neural phenotype. For this reason, stem cells can furnish potential material for cell replacement therapy. In summary, this thesis provides a new and unexplored molecular-mechanism for understanding the appearance and maintaining Krabbe disease hallmark. This work is the first description of a potential Connexin43 involvement in oligodendrocytes maturation and psychosine-inducted toxicity.

SOMMARIO

La leucodistrofia di Krabbe o a cellule globoidi è un disturbo autosomico recessivo a carico del gene codificante per galactosilcerebrosidase (GALC). GALC è coinvolto nel catabolismo di due lipidi molto prodotti nel processo di formazione della guaina mielinica: galactosilceramide e psicosina. A differenza delle classiche lipidosi, galactosilceramide non si accumula nel cervello di pazienti, mentre ciò non accade per la psicosina, che determina la scomparsa degli oligodendrociti cui fa seguito una severa demielinizzazione e neurodegenerazione invalidante. Questo studio è stato rivolto all'approfondimento di alcuni meccanismi molecolari tramite i quali la psicosina determina l'induzione dell'apoptosi in precursori di oligodendrociti e in fibroblasti con e senza mutazione a carico del gene *GALC*. Poiché, fino ad oggi, non sono mai state condotte ricerche mirate a valutare l'espressione e la localizzazione di proteine che svolgono ruoli essenziali nell'omeostasi e nella comunicazione cellulare mediata dalle gap-junctions di membrana in precursori di oligodendrociti privi o dotati della deficienza di GALC, l'eventuale coinvolgimento della Connessina43 è stato considerato. I meccanismi che interferiscono nel processo di maturazione degli oligodendrociti e la possibilità di impiegare cellule mesenchimali staminali ottenute da tessuto adiposo in malattie neurodegenerative sono state, inoltre, esaminati. I risultati hanno dimostrato che la psicosina esogena determina effetti differenti sui modelli utilizzati che dipendono dalla diversa sensibilità delle singole tipologie di cellule alle varie concentrazioni di lipide usato. La psicosina ha influenzato primariamente la proliferazione e la crescita cellulare, determinando l'attivazione di un programma apoptotico mediando l'attivazione della caspase-3 e alterando i livelli di PTEN e PI3K, coinvolti nella crescita e sopravvivenza cellulare. In parallelo, la presenza della psicosina ha determinato nei modelli cellulari impiegati la proteolisi della procaspase-3, l'aumento di espressione di Bad e di p53 e l'attenuazione dell'attivazione di NF-kB. Gli effetti più severi sono stati registrati nei precursori degli oligodendrociti piuttosto che nei fibroblasti. La presente ricerca ha dimostrato, per la prima volta, che i precursori di oligodendrociti esprimono Connessina43, normalmente espressa negli astrociti, ma non negli oligodendrociti maturi. Inoltre, gli alti livelli intracitoplasmatici di Connessina43 giustificano le differenze dose-dipendenti del danno indotto dalla psicosina e il mancato sviluppo del fenotipo maturo dei precursori di oligodendrociti. Da quest'ultima considerazione è nata la curiosità di indagare sulla probabilità di rimpiazzare gli oligodendrociti non maturati con cellule mesenchimali staminali ottenute da tessuto adiposo (AT-MSCs). Gli studi *in vitro,* descritti in questo lavoro, hanno dimostrano che le AT-MSCs possono essere indirizzate dal "milieu" extracellulare verso un fenotipo neurale e, nello specifico, oligodendrocitario. Benché i risultati evidenzino che le AT-MSCs possano fornire un potenziale materiale per la "cell replacement therapy", nuovi esperimenti sono necessari per confermare questa ipotesi. In conclusione, i risultati presentati in questa tesi offrono un meccanismo molecolare nuovo e inesplorato per comprendere gli aspetti

patognomonici della leucodistrofia di Krabbe. Inoltre, suggeriscono per la prima volta un potenziale coinvolgimento della Connessina43 nella mancata maturazione degli oligodendrociti e nella tossicità indotta dalla psicosina e forniscono i preliminari per una "cell replacement theraphy" applicata a patologie neurodegenerative.

.

INTRODUCTION

"J'ai la conviction que quand la physiologie sera assez avancée, le poète, le philosophe et le psysiologiste s'entendront tous"[1]

Claude Bernard (1865)

[1] Conclusive statement of a short, popular article entitle "Etude sur la physiologie du Coeur", published in the "Revue des Deux Mondes".

OLIGODENDROCYTES

The cells of the nervous system have been divided into two main categories: neurons and glia (Kettenmann and Verkhratsky, 2008).

Glial cells are divided into two major categories: macroglia and microglia. The macroglia (also known as neuroglia) are made up of oligodendrocytes, astrocytes, NG2+ cells (polydendrocytes) and ependymal cells. The microglia are derived from myeloid-monocytic cells and are the resident macrophages of the CNS (Tambuyzer et al., 2009).
Even if these scholastic description is clear today, glial cells identification and characterization required a very long, confusing and often controversial process (Somjen, 1988).
In 1856, the German physician Rudolph Virchow, also known as the "Pope of pathology" (Kettenmann and Ransom, 2005; Magner, 2002), introduced the term *neuroglia* for describing a "*connective substance ... in which nervous system elements are embedded*" and referred to it as "*nervenkitt*" (or nerve putty), as the word etymology suggest (from archaic Greek, meaning something sticky or clammy). Long time, this description was a kind of dogma and led to consider neuroglia an acellular connective tissue rather than made up of cells as it is really.
The first appearance in literature of cells that later were known as oligodendrocytes was in 1900, when W. Ford Robertson described a group of small cells with few processes that he believed to be mesodermal in origin and subsequently named them mesoglia.
Despite this initial description, the oligodendrocytes were not considered, or at least ill defined, for the next two decades, when, in 1911, the eminent neuropathologist Ramòn y Cajal defined a group of cells distinct from both neurons and astrocytes with the name of "the third element of Cajal".

The differentially stain of the cell types of the third element of Cajal was made by Rio Hortega, in 1918 and the introduction of the terms microglia and oligodendroglia was made possible (Gill and Binder, 2007).Synthetically, the techniques of metallic impregnation were essential for characterizing the major glial cell types:

- gold impregnation by Ramon y Cajal (Ramon y Cajal S,1913) identified the astrocyte among neuronal cells, as well as the third element which was not impregnated by this technique;
- silver carbonate impregnation, developed by Rio Hortega, found two other cell types, the oligodendrocyte (Rio Hortega DP, 1928), first called interfascicular glia, and another cell type distinguished from the macroglial cells (i.e., macroglia), and called microglia (Rio Hortega DP, 1921).

Rio Hortega reported that these cells were present in large numbers in all regions of the CNS but predominantly in the white matter, frequently located near neurons, blood vessels and in series along nerve fibers. Furthermore, he suggested that the membrane around central myelinated fibers, identified by Cajal, was a derivative of oligodendroglia cells with functions similar to those of Schwann cells of peripheral myelinated fibers.
The electron microscopy confirmed this assertion in the 1960s.

Rio Hortega later went on to define perineuronal or interfascicular oligodendrocytes of four classes (Rio Hortega DP, 1928) according to their number of processes and morphology (also described in Bunge et al., 1961), a classification which remains largely unchanged.
According to their morphology and the size or thickness of the myelin sheath, Butt et al. (1995.) distinguished four types of myelinating oligodendrocytes: from small cells supporting the short, thin myelin sheaths of 15–30 small diameter axons (type I), through intermediate types (II and III), to the largest cells forming the long, thick myelin sheaths of 1–3 large diameter axons. At the electron microscopic, the cytoplasmic densities and the clumping of nuclear chromatin gave a spectrum of morphological variations, thus light, medium, and dark oligodendrocytes were described (Mori and Leblond, 1970). Based on labelling with tritiated thymidine of the corpus callosum of young rats, light oligodendrocytes seem to be the most actively dividing cells, medium progressively in maturation, and dark with the densest cytoplasm.

Functions of oligodendrocytes

The main function of oligodendrocytes is the formation of a myelin sheath around most of axons in the central nervous system (CNS), while Schwann cells do it in periferal nervous system (PNS). Traditionally, oligodendrocytes support myelinated axons and promote action potential conduction by insulating effects of the multilayered myelin sheath. Among the three main types of glial cells in the CNS, oligodendrocytes have the most special and intimate interactions with the axons: the myelin sheet is an extension of their plasma membrane which, enriched in lipids, ensheats the axon (Fig. 3).

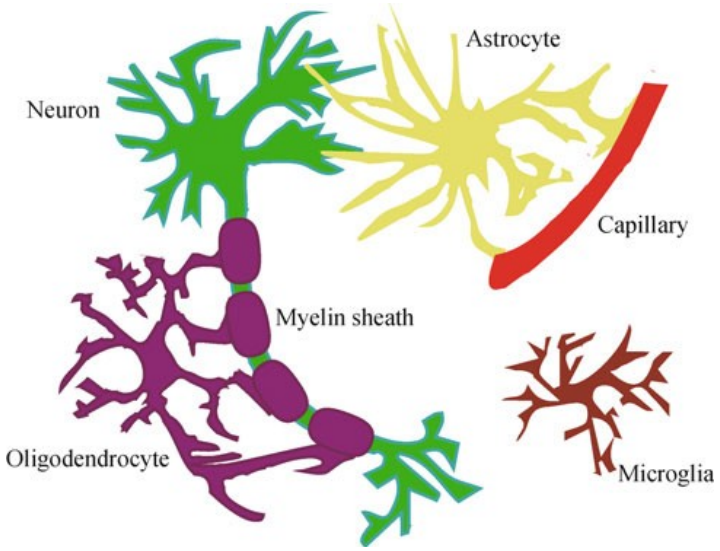

Fig. 3. The intimate structural interaction between oligodendrocytes and neurons
Oligodendrocytes can wrap neighboring axons through multilayered myelin sheath.
[from Liu and Zhou, 2013]

In contrast to other membranes, composed by 30–50% lipid, oligodendrocytes-producted myelin is approximately formed by 70% lipid and 30% protein (Norton and Cammer, 1984). This peculiarity gives to myelin-forming cell an important function in maintaining low capacitance, enveloping high resistance membrane and insulating electrically the axon; thus the rapid and efficient propagation of impulses is allowed. In addition, myelin provides trophic support to the axon and maintains its long-term integrity.

Therefore, oligedendrocytes membranes extend their function beyond providing insulation: they facilitate tri-directional communication between the neuron, the myelinating cell, and the environment (Nave and Trapp, 2008).

Recent findings obtained by genetic ablation of oligodendrocytes in adult mice strongly suggest that oligodendrocytes are necessary for axonal integrity under physiological conditions (Ghosh et al., 2011; Pohl et al., 2011; Oluich et al., 2012). These studies were carried out by three independent research groups, using distinct methods, and the data obtained seem in agreement with the hypothesis that oligodendrocytes can support their associated axons and maintain long-term functional integrity through mechanisms other than myelination (Nave and Trapp, 2008; Nave, 2010a and 2010b).
These scientific approaches could be useful for explaining the observed relationship between the intact myelin sheath, oligodendrocytes loss and the axon damages in several neurological diseases such as multiple sclerosis (MS) and leukodystrophies (Feigenbaum et al., 2000; Schiffmann and van der Knaap, 2004; Trapp and Nave, 2008). The exact molecular processes underlying these damages are still in debate.

Mugnaini E. suggested another remarkable link in 1986. In a review on cell junctions of the CNS supporting cells, the author comments: "Cell junctions require careful analysis because they reflect not only the biology of individual cells, but also their sociology; that is, the cooperativity with other cells, and the relation to the environment." Morphological studies *in situ* have shown that astrocyte makes gap junctions localized between cell bodies, between processes and cell bodies, and between astrocyte end-feet that surround brain blood vessels (Yamamoto et al., 1990). *In vitro*, junctions between astrocytes were observed too (Fischer and Kettenmann, 1985; Venance et al., 1997). Gap junctions occur also between oligodendrocytes, as observed *in situ* (Butt and Ransom. 1989; Robinson et al., 1993; Sotelo and Angaut, 1973) and *in vitro* (Venance et al., 1995.), but less frequently.
Moreover, astrocyte-to-oligodendrocyte gap junctions were identified between cell bodies, cell bodies and processes, and astrocyte processes and the outer myelin sheath (Waxman and Black, 1984; reviewed in Giaume and Venance, 1995). Thus, glial cells form a generalized glial syncytium (Mugnaini, 1986) also called "panglial syncytium," that is a large glial network, radially extended (Rash et al., 1997) (Fig. 4).

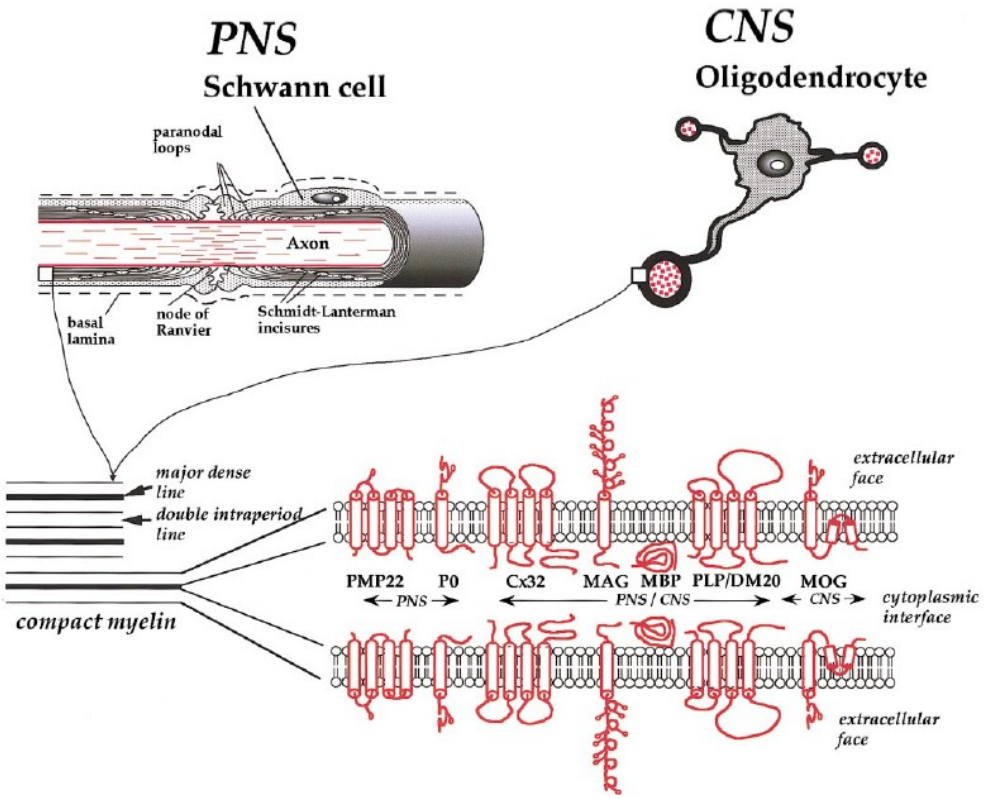

Fig. 4. Myelinating glial cells, myelin structure, and composition in the PNS and in CNS
In the PNS, the myelinating Schwann cell myelinates only one segment of axon (top left corner), whereas in the CNS (top right corner), the oligodendrocyte is able to myelinate several axons. The myelin proteins are schematically described; they differ between PNS and CNS.
[Adapted from Pham-Dinh, 1998]

Gap junctions are channels that link the cytoplasm of adjacent cells and permit the intercellular exchange of small molecules with a molecular mass of 1–1.4 kDa, including ions, metabolites, and second messengers (Bruzzone and Ressot, 1997; Giaume and Venance, 1995).
Homologous coupling could serve to synchronize the activities of neighboring cells that serve the same functions. This type of junction acts as a functional network: the different channels groups vary in their permeability, voltage sensitivities, and potential for modulation by intracellular factors (Giaume and Venance, 1995).

Heterotypic coupling, instead, may also serve to coordinate the activities of the coupled cells by providing a pathway for the selective exchange of molecules below a certain size. Heterocoupling between astrocytes and oligodendrocytes has been proposed to serve K^+ buffering around myelinated axons (Li, Hertzberg and Nagy, 1997; Zahs et al, 1998).
In this way, the glial network could have new and unexplored functions, far away the early and simple role as "nerve glue".

Differentiation of oligodendrocytes

Oligodendrocytes derived from neuroectodermal cells within the subventricular zone and their process for differentiating is extremely fascinating.

Oligodendrocytes respond to axonal signals (Kidd et al., 1990; Scherer et al., 1992) for their maturation program, although they can also differentiate in culture and *in vivo* in the absence of neurons and viable axons, respectively (Dubois-Dalcq et al., 1986; Mirsky et al., 1980; Ueda et al., 1999).

Before their final maturation involving myelin formation, the oligodendrocytes go through a default pathway by an intrinsic program controlling the timing of development (Temple and Raff, 1986) and external signals promoting or inhibiting oligodendrocyte differentiation and CNS myelination (Coman et al., 2005; Nave and Trapp, 2008; Simons and Trajkovic, 2006).

The many stages of development can be identified by different methods:

- the observation of morphology;
- the analysis of specific components expressed on plasma membrane surface, at distinct time points. Several of those are glycosphingolipids (GSLs) (Jackman et al, 2010);
- the visualization of spectrum with electron microscopy.

For characterizing cell development, morphological criteria alone are often insufficient both *in vivo* and *in vitro*, while the identification based on the expression of various specific components (antigenic markers) and the mitotic and migratory status of these cells seems to be more selective and efficient for distinguishing all phenotypic stages, both *in vivo* and *in vitro*.

Based on it, the different developmental stages of the oligodendrocytes lineage have been well-characterized (Pfeiffer et al., 1993):

- Early oligodendrocytes progenitors are bipolar, migratory, proliferative cells recognized by monoclonal antibody (mAb) A2B5, against surface gangliosides, GD3, GT3, and O-acetylated GT3, that are subsequently downregulated as the progenitors differentiate into an immature oligodenrocytes. These latter proliferate *in vitro*, in response to growth factors such as fibroblast growth factor (FGF) (Bansal, 2002) and platelet derived growth factor (PDGF) (Yan and Rivkees, 2002; Gard and Pfeiffer, 1993; Milner, 1997; Hardy and Reynolds, 1993; Raff et al., 1988; Richardson et al, 1988).

- Late progenitors (Prooligodendroblasts,Pro-OL) elaborate more processes. In addition to A2B5 immunoreactivity, they express POA, an unidentified sulfated glycolipid recognized by the monoclonal antibody O4 (Bansal et al., 1992; Bansal et al.,1989; Bansal et al., 1999; Gard and Pfeiffer, 1990; Sommer and Schachner, 1982). At this stage, cells are less motile (Orentas

and Miller,1996), or even post migratory (Pfeiffer et al., 1993), and lose their mitogenic response to PDGF (Gao et al., 1998; Hart et al., 1989; Pringle and Richardson,1993). Probably platelet derived growth factor receptor alpha (PDGF-Rα) is recruited into lipid rafts as oligodendrocytes shift from a proliferative phenotype in a differentiation program, changing the role of PDGF from a mitogen to a survival factor (Baron et al., 2003).

- Transient "Pre-GalC" stage ologodendrocytes, when the cells stop proliferation and are on the verge of entering terminal differentiation marked by immunoreactivity against R-mAb (a monoclonal antibody that recognizes GalC and sulfatide) (Bansal and Pfeiffer, 1992; Bansal and Pfeiffer, 1989; Bansal et al., 1989; Ranscht, 1982).

- Mature oligodendrocytes, that form 'myelin-like' membranes *in vitro* and myelin sheaths that enwrap axons *in vivo*. They represent the last differentiation stage, developing a more complex morphology and begin to abundantly synthesize galactosylceramidase (GalC) (recognized by O1 mAb in addition to R-mAb) and sulfatide (O4 at this stage recognizes sulfatide in addition to pro-oligodendroblast antigen; POA).

Myelin proteins, such as 2′-3′-cyclic nucleotide 3′ phosphohydrolase (CNP), myelin-associated glycoprotein (MAG), myelin basic protein (MBP), myelin/oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP) are also sequentially expressed during the progressive morphological maturation of cells.
Several studies suggest that glicosphingolipids can interfere in terminal differentiation of the oligodendrocytes: the treatment with R-mAb, that recognizes both GalC and sulfatide, inhibits terminal differentiation of late progenitors to GalC+ oligodendrocytes (Fig. 5), suggesting that these glicosphingolipids can act as sensors and transmitters of environmental information (Bansal and Pfeiffer, 1989).

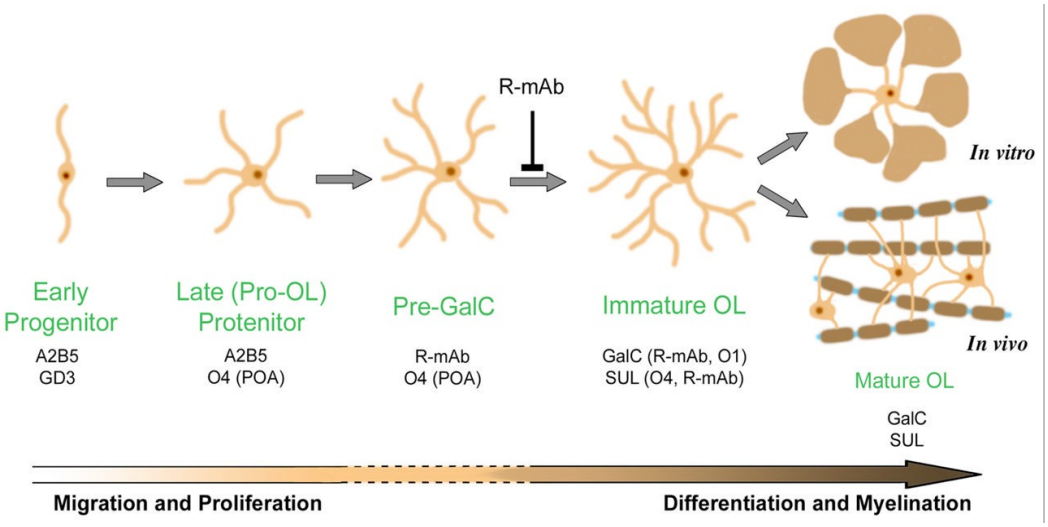

Fig. 5. Representation of oligodendrocyte differentiation pathway
Expression of specific lipids, protein markers, change in morphology, migratory and proliferative capacity of cells are hallmarks of each stage of oligodendrocyte maturation. Lineage progression can be reversibly blocked in culture by treatment with R-mAb antibody.
OL: oligodendrocyte; POA: pro-oligodendroblast antigen; GalC: galactocerebroside; SUL: sulfatide; GD3: ganglioside. A2B5, O4, O1, R-mAb: names of antibodies.
[Adapted by Taylor et al. 2004 from Pfeiffer et al., 1993;]

Specifically, antibodies against sulfatide, but not GalC, produced arrest in terminal differentiation, and upon removal of the antibody, differentiation proceeded normally (Bansal et al., 1999).

Different studies analyzed the interference of lipid-mediated signaling in oligodendrocytes maturation suggesting that lipids are involved in maintaining the myelin integrity (Hirahara et al., 2004; Marcus et al., 2000; Shroff, 2009; Dyer and Benjamins, 1988; Dyer and Benjamins 1990).

The differentiation stages of olidodendrocytes are regulated by intrinsic and extrinsic factors that orchestrate the myelination phenomena, even if the signaling pathways are far from complete.
Differentiation is predominantly regulated on the level of gene transcription and protein translation and much progress has been made in the characterization of these intrinsic factors. On the level of gene transcription, the transcription factors promoting maturation are: Olig1, Olig2 and Nkx2.2 and Sox10 (Liu and Casaccia, 2010; Miron et al., 2011). Especially relevant for the initiation of differentiation is the concomitant expression of Olig2 and Nkx2.2 (Zhou et al., 2001), while the others transcription factors are expressed in all stages of oligodendrocytes development. Indeed, Hes5, Id2, Id4, Sox5 and Sox6, strongly inhibit the maturation program (Liu and Casaccia, 2010; Miron et al., 2011). In addition, expression of other proteins can be regulated by histone deacetylation. Thus, the expression of stathmin is repressed

by this mechanism during oligodendrocytes differentiation (Liu et al., 2003) thereby promoting microtubule polymerization. Interesting studies investigated intrinsic mechanism by miRNAs that are small non-coding RNAs processed from larger precursor RNAs (Dugas et al., 2010; Zhao et al., 2010).
Two main classes of extrinsic factors act on oligodendrocytes maturation program: long-range signals such as growth factors; short-range signals such as extracellular matrix (ECM) and cell adhesion molecules.
A schematic representation of extracellular factors is given in Table 1, focusing on the principal source of stimulus.

			Predominant source	Effect on oligodendrocyte lineage
Factors	Soluble factors	Adenosine	Neurons	promotes differentiation
		Glutamate	Neurons	promotes differentiation
		PDGF-AA	Astrocytes	promotes migration and proliferation; inhibits differentiation
		FGF-2	Astrocytes	promotes migration and proliferation; inhibits differentiation
		IGF-1	Astrocytes	promotes differentiation
		CNTF	Astrocytes	promotes differentiation
		LIF	Astrocytes	promotes differentiation
		BMP	Astrocytes	inhibits differentiation
	Membrane proteins	LINGO-1	Neurons	inhibits differentiation
		Jagged	Neurons	inhibits differentiation
		PSA-NCAM	Neurons	inhibits interaction with axon
		Neuregulin-1	Neurons	promotes myelination
	ECM molecules	Laminin-2	Neurons	promotes differentiation
		Fibronectin	Astrocytes	promotes migration and proliferation; inhibits differentiation
		Tenascin C	Astrocytes	inhibits migration and differentiation
	Other factors	Electrical activity	Neurons	promotes differentiation

Table 1 Neuronal and astrocytic factors regulate oligodendrocytes behavior and differentiation

As it is possible understanding in Table 1, many growth factors are involved in the proliferation, differentiation, and maturation of the oligodendrocyte lineage (Barres et al., 1994; Canoll et al, 1999; Canoll et al., 1996; Hardy and Reynolds, 1991; Hardy and Reynolds, 1993;McKinnon et al., 1990; McMorris et al., 1988). Most of these studies have been performed *in vitro*, because *in vivo* multiple factors may act in

concert to achieve the regulation of the complex process of oligodendrocyte development and myelination.
Among the growth factors:

- PDGF is synthesized during development by both astrocytes and neurons (Mudhar et al., 1993, Yeh et al., 1991). *In vitro*, PDGF is a survival factor for oligodendrocyte precursors (Grinspan and Franceschini, 1995), and a potent mitogen. The blockade of the intracellular signaling pathways from the PDGF receptor to the nucleus (Hart et al., 1989) controls the number of cellular divisions (Gao et al., 1998). The PDGF-α receptors disappear at the O4+ stage of oligodendrocyte maturation (Ellison and de Vellis, 1994; Nishiyama et al., 1996). PDGF also stimulates the motility of oligodendrocyte progenitors *in vitro* and is chemoattractive.

- Basic FGF (bFGF), also called FGF 2, is a mitogen for neonatal oligodendrocyte progenitors (Eccleston, 1984). It upregulates the expression of PDGFR-α and therefore increases the developmental period during which oligodendrocyte progenitors or preoligodendrocytes are able to respond to PDGF (McKinnon, 1990). Preoligodendrocytes can even revert to the oligodendrocyte progenitor stage when cultured with both PDGF and bFGF (Bogler et al., 1990).

- Insulin-like growth factor-I (IGF-I) stimulates proliferation of both oligodendrocyte progenitors and preoligodendrocyte $O4^+$ cells, and IGF receptors is present on cells of the oligodendrocyte lineage (McMorris et al, 1988).

- Glial growth factor (GGF), a member of the neuregulin family of growth factors generated by alternative splicing, including Neu, heregulin, and the acetylcholine receptor-inducing activity (ARIA), is a neuronal factor, mitogenic on oligodendrocyte precursors (Canoll et al., 1996, Milner et al., 1997); it is also a survival factor for these cells. It delays differentiation into mature oligodendrocytes (Canoll et al., 1999). Neuregulin is a strong candidate as an axon-derived promoter of myelinating cell development (Barres and Raff, 1999).

- Ciliary neurotrophic factor (CNTF) can act as comitogen with PDGF; it also promotes oligodendrocyte survival *in vivo* (Barres et al., 1993).

A complete description is represented in Fig. 6.

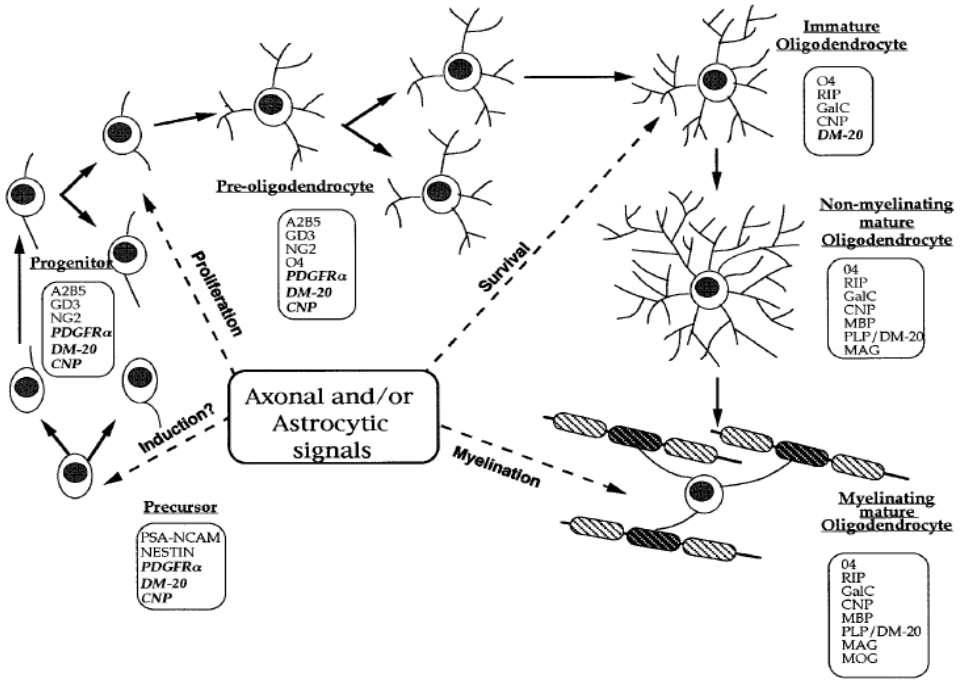

Fig. 6. Scheme of oligodendrocytes maturation under neuronal and astrocytic factors
Stage-specific markers are boxed. RNAs are in italics.
[From Lubetzki et al., 1993; Hardy and Reynolds,1993; Pfeiffer et al.,1993]

In order to give an exhaustive description of this finely regulated differentiation program, another factor influences glial cell maturation leading to myelin formation: 3,3',5-triiodothyronine (T_3). In T_3-enriched culture medium, oligodendrocyte progenitors stop the cell division and differentiation, although this process may occur even in absence of this signal. Thyroid hormone may be involved in the timing of differentiation of this cell population (Barres et al., 1994) and it promotes morphological and functional maturation of post-mitotic oligodendrocytes, thus determining the number of mature oligodendrocytes (Baas et al., 1997; Ibarrola and Rodriguez-Pena, 1997).

Recent advances in evaluation of parameter interfering during oligodendrocytes development indicate different neurotransmitter receptors involved in processes other than nerve conduction, as trophic functions in glial and neuronal differentiation

Oligodendrocyte progenitors are equipped with a variety of ligand- and voltage-gated ionic channels. It is known that glutamate is the most abundant excitatory neurotransmitter in the mammalian brain, acting on its ionotropic receptors. Glutamate is involved in the shaping of the oligodendrocyte population. The main ionotropic glutamate receptors expressed by oligodendrocytes belong to the dl-a-amino-3-hydroxy-5-methylisoxazole- 4-propionic acid (AMPA) and kainate classes (Matute, 1998). It has been shown that non-NMDA glutamate receptor agonists are able to inhibit oligodendrocytes progenitor proliferation in cell cultures (Gallo et al., 1996). In cerebellar slice cultures, glutamate is an antimitotic signal at all proliferative phases of the oligodendrocyte lineage whereas in the same context,

astrocyte proliferation and number are not affected. The glutamate effects are receptor and cell specific, because they are selectively mediated through AMPA receptors (Yuan et al., 1998).

The dopamine D3 receptor (D3R) has been found to be expressed by precursors and immature oligodendrocytes, but absent in mature oligodendrocytes (Bongarzone et al., 1998). Another dopamine receptor, D2R, is present in a subset of mature interfascicular oligodendrocytes in the rat corpus callosum (Howard et al., 1998).

GABA-A receptors have also been reported in oligodendrocytes (Berger et al., 1992).

Oligodendrocytes express opioid receptors, μ-receptors are apparent at the earliest stages of oligodendrocyte development, while κ-receptors are detected later at the time that MBP is expressed (Kolodny, 1993). There is a proliferative response to μ-receptor stimulation.

These changes in neurotransmitter receptors expression could be linked to additional function of their specific ligands during orchestration of oligodendrocytes differentiation, but furthermore studies are necessary.

Oligodendrocytes and neurodegeneration

The significance of oligodendrocyte under pathological conditions especially in neurodegenerative diseases has not been fully recognized, even if they are an important player in normal brain functions.

Emerging evidence demonstrates that dysfunction of oligodendrocytes can induce pronounced neurodegeneration, although the precise molecular and cellular mechanisms still need to be unraveled. Thus the evaluation of this potential role of oligodendrocytes became more and more interesting and considered in last years.

For example, roles of oligodendrocytes in protein aggregation related to neurodegenerative diseases, such as tauopathies and synucleinopathy, have been documented. Oligodendrocytes and myelin disturbance and their possible roles in the initiation and progression of Alzheimer disease (AD) and Huntington's disease (HD) have attracted more attention from the scientific community, recently (Desai et al., 2010; Mitew et al., 2010; Valenza et al., 2010; Desai et al., 2011; Valenza and Cattaneo, 2011).

In summary, oligodendrocytes are now considered as multi-functional glial cells in the central nervous system and they actively play important roles under both physiologic and pathological conditions (Fig. 7).

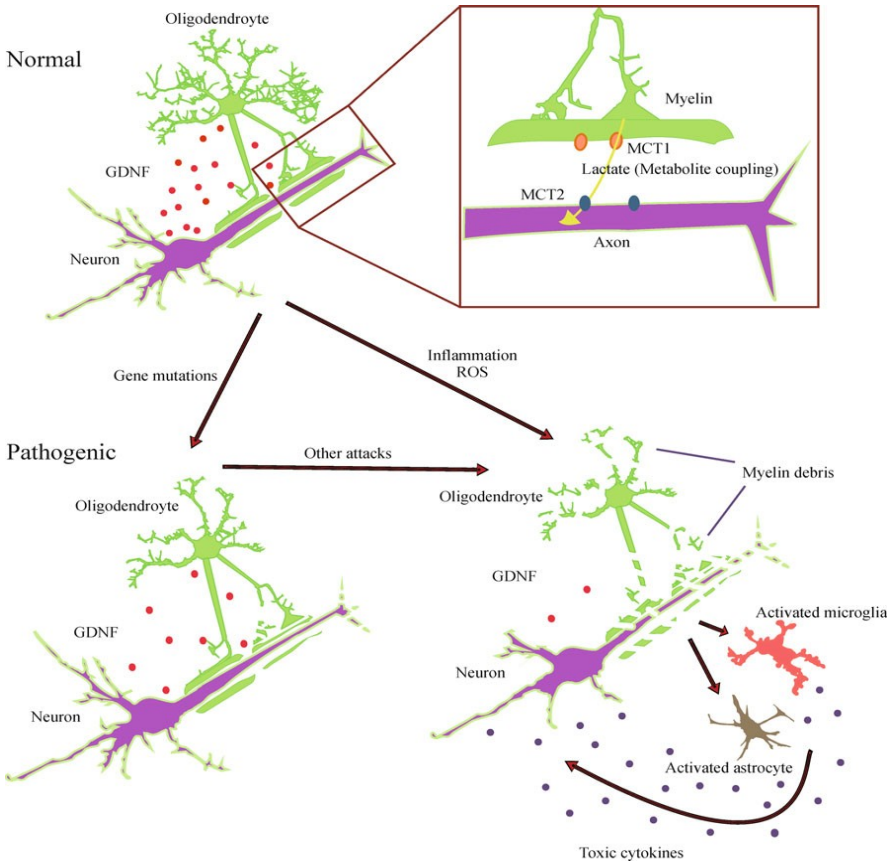

Fig. 7. Mechanisms of neurodegeneration induced by oligodendrocyte dysfunction
Series of insults can cause dysfunctions or death of oligodendrocytes that in turn results in axon neurodegeneration through myelin breakdown, neuroinflammation, lactate uncoupling, decreased neurotrophic factors secretion and other unknown mechanisms. [From Liu and Zhou, 2013]

Oligodendrocyte disturbances are associated with major diseases of the nervous system.

The knowledge of basic principles occurring in oligodendrocyte apoptotic loss could offer new avenues for therapeutic interventions. In a review, Bradl and Lassmann (2010) clarified the goals obtained in the last decades and offered a well-detailed work on oligodendrocytes biology, with a little accent to the most researched signals involved in oligodendrocytes suffering and death.

Due to the combination of a high metabolic rate with its toxic byproducts, high intracellular iron, and low concentrations of the antioxidative glutathione, oligodendrocytes are particularly vulnerable to oxidative damage (Thorburne and Juurlink, 1996; Juurlink, 1997). Hence, oxidative damage is a common contributor to oligodendrocyte loss under many pathological conditions like multiple sclerosis (MS) and ischemia. It can act in concert with the sphingomyelinase/ceramide pathway: ceramide is the core component of sphingolipids, the major lipid components of myelin sheaths (Baumann and Pham-Dinh, 2001). Ceramide is released by the action of sphingomyelinase that is normally inactive, but becomes activated in response to oxidative stress (Jana and Pahan, 2007; Singh et al., 1998),

inflammatory mediators (Singh et al., 1998; Brogi et al., 1997; Testai et al., 2004), injury or infection (Schenck et al., 2007). Once released within oligodendrocytes, ceramide can activate pro-apoptotic signaling cascades eventually culminating in oligodendrocyte loss (Brogi et al., 1997; McTigue and Tripathi, 2008).
Oligodendrocytes express an arsenal of molecules rendering them susceptible to excitotoxic cell death (Domercq et al., 2007; Li and Stys, 2000; Matute et al., 1997;McDonald et al., 1998; Sanchez-Gomez and Matute, 1999). They possess AMPA (Tanaka et al., 2000), kainate (Sanchez-Gomez and Matute, 1999; Alberdi et al., 2006), and NMDA (Karadottir et al., 2005; Micu et al., 2006; Salter and Fern,2005) receptors which make them vulnerable to glutamate toxicity. Moreover, they have the ATP receptor P2X7 (Matute et al., 2007) that predisposes them to the damaging action of sustained levels of extracellular ATP (Matute et al., 2007).

Oligodendrocyte loss can occur by exposure to inflammatory cytokines. For example, tumor necrosis factor-α (TNF-α) can induce apoptosis of oligodendrocytes by binding to their p55 TNF receptor (Jurewicz et al., 2005).

The situation is more complex in the case of interferon gamma (IFNγ). This cytokine is highly toxic for actively proliferating, much less for immature, and not at all for mature oligodendrocytes (Horiuchi et al., 2006). Besides these direct actions, inflammatory mediators may damage oligodendrocytes indirectly through stimulation of radical production from microglia and possibly astrocytes.

Oxygen- and nitric oxide-radicals are particularly toxic for mitochondria through interaction and blockade of various proteins of the respiratory chain (Mahad et al., 2008; Smith and Lassmann, 2002). Others studies on changes of gene expression in glia cells showed that many different pro-inflammatory cytokines can induce mitochondrial injury (Lisak et al., 2009). As mentioned above, oligodendrocytes are particularly vulnerable to oxidative damage and mitochondrial injury. Oligodendrocytes are damaged in certain toxic states, which interfere with mitochondrial function. Examples for this are the selective oligodendrocyte apoptosis and demyelination induced by cuprizone, a copper chelator interfering with complex IV of the mitochondrial respiratory chain (Torkildsen et al., 2008), and by the intoxication with cyanides blocking the respiratory chain at the level of complex IV too (Carelli et al., 2002; Hirner,1969).

All these mechanisms described above do not destroy oligodendrocytes specifically, but may impair function and viability of other cells, such as neurons and astrocytes.
However, oligodendrocytes and their myelin sheaths are in general more susceptible to damage than other cellular components of the nervous system. This explains the so-called ‘‘bystander damage’’ of myelin and oligodendrocytes observed in many inflammatory disease states, in which the immune reaction is not specifically directed against these cells (Wisniewski and Bloom, 1975). In fact, demyelination and oligodendrocyte death is a common feature of inflammatory white matter

lesions, both in humans and experimental models. A particularly illustrative example is Devic's neuromyelitis optica (NMO). This disease has been originally classified as an inflammatory demyelinating disease due to the presence of widespread primary demyelination in the spinal cord and optic nerves (Lucchinetti et al., 2002). But, in this case, immunological studies provide clear evidence that in NMO the primary targets of the pathogenic immune (autoantibody) response are not oligodendrocytes, but astrocytes (Lennon et al., 2004; Lennon et al., 2005). Time course studies on lesion development in NMO patients revealed that astrocytes are destroyed first, but that this is followed by profound demyelination and oligodendrocyte, axons and nerve cells destruction (Misu et al., 2007; Misu et al., 2008). It is currently unresolved whether, in this disease, oligodendrocyte injury is only a bystander reaction of the inflammatory process or whether a specific disturbance of the homeostatic interaction between astrocytes and oligodendrocytes plays an additional role.

Besides that through non-specific bystander mechanisms, oligodendrocytes can be destroyed by specific, cell selective immune mechanisms. Autoantibodies directed against an epitope on the extracellular surface of myelin or oligodendrocytes can induce demyelination through activation of complement or through their recognition by Fc-receptors of activated macrophages. The most compelling examples for such autoantibodies are those directed against myelin oligodendrocyte glycoprotein (MOG), (Linington et al., 1988) and galactocerebroside (Dubois-Dalcq et al., 1970). Antibody-mediated demyelination is an important mechanism in models of autoimmune encephalomyelitis and seems to play a role also in a subset of patients with MS-like inflammatory demyelinating diseases (Lucchinetti et al., 2000, O'Connor et al., 2007). Similarly, cytotoxic T-lymphocytes, directed against a myelin or oligodendrocyte antigen, or a foreign (e.g., virus) antigen expressed in oligodendrocytes, can induce oligodendrocyte apoptosis, followed by selective demyelination (Huseby et al., 2001; Na et al., 2008; Saxena et al., 2008).

Myelinating cells and diseases

The most frequent disease involving oligodendrocytes is multiple sclerosis. It is characterized by a loss of myelin in defined areas of brain and spinal cord that leads to an impairment of axonal conductance. Recovery can occur due to re-myelination, but the continuous occurring relapses could be due to continuous neurodegeneration. The primary cause for the loss of oligodendrocytes is yet unknown. It is evident that the demyelinated region contains inflammatory cells such as infiltrating lymphocytes and macrophages and activated microglia. These cells might potentiate or even initiate the damage cascade.

Other inherited myelin disorders of the central nervous system are Pelizaeus-Merzbacher and Pelizaeus-Merzbacher-like diseases and other forms of leukodystrophies. Most of the genetically determined pathologies are associated with mutations in myelin proteins or connexins, the molecular entities forming gap junctions. Similarly, mutations in Schwann cell myelin or gap junction proteins lead

to neuropathies such as the Charcot–Marie–Tooth disease**.** Therefore the peripheral myelin formation is essential for the survival of vertebrates (Kettenmann and Verkhratsky, 2011).

The complete sequencing of genomes has opened up new and powerful approaches to study oligodendrocytes. Only a handful of genes have been identified that, when mutated, cause myelin diseases in human (Table 2).

Gene	Gene locus (humans)	Human disease	Mouse model	Reference
Proteolipid protein 1 (Warshawsky et al.,2005)	Xq22	Pelizaeus_Merzbacher disease (PMD)	jimpy, jimpy^{msd}, rumpshaker, plp1knockout	(Garbern, 2007)
Myelin basic protein (MBP)	18q22-qter	18q- syndrome (hypomyelination detected by MRI)	shiverer mbp knockout	(Gay et al., 1997; Loevner et al., 1996)
Connexin 47 (Uhlenberg et al., 2004)	1q41_42	Pelizaeus-Merzbacher-like disease, autosomal recessive	cx47 knockout	(Henneke et al., 2008; Salviati et al., 2007; Uhlenberg et al., 2004)
Connexin 32	Xq13.1	Charcot-Marie-Tooth CMT1X with central conduction slowing	cx32 knockout	(Murru et al., 2006; Seeman et al., 2001)
Eukaryotic initiation factor 2B (eIF2B)	12q24.3 14q24 1p34.20 2p23.3 3q27	Vanishing white matter; childhood ataxia with CNS hypomyelination(Labauge et al., 2007)		(Labauge et al., 2007; Leegwater et al.,2001; Maletkovic et al., 2008)
Aspartoacylase (ASPA)	17pter _ p13	Canavan disease tremor	(rat) aspa knockout	(Moffett et al., 2007; Namboodiri et al., 2006)
Galactocerebrosidase (Gard et al., 1995)	14q31	Globoid cell leukodystrophy (Krabbe's)	twitcher	(Suzuki, 2003)
ABCD1	Xq28	X-linked adrenoleukodystropy (X-ALD)	abcd1 knockout	(Moser et al., 2007)
Quaking (Qk1)	6q25_27	Susceptibility to schizophrenia	quaking	(Aberg et al., 2006)
Nogo receptor (NGR) eticulon 4 receptor	22q11	Susceptibility to schizophrenia	ngr knockout	(Sinibaldi et al., 2004)

Table 2. Oligodendrocyte gene mutations associated with CNS myelin diseases
[From Nielsen, Lau and Hudson, 2010]

KRABBE DISEASE

Lysosomal storage diseases

In 1955, De Duve discovered the lysosome, degradative organelle present inside cell cytoplasm. The inner lysosomal milieu has an acid pH and it contains a wide array of enzyme, at least 50 type of hydrolases that explain an extremely selective catalytic activity on different substrate (Fig. 8). Lysosomes have an important role in cell metabolism and consequently in cell function.

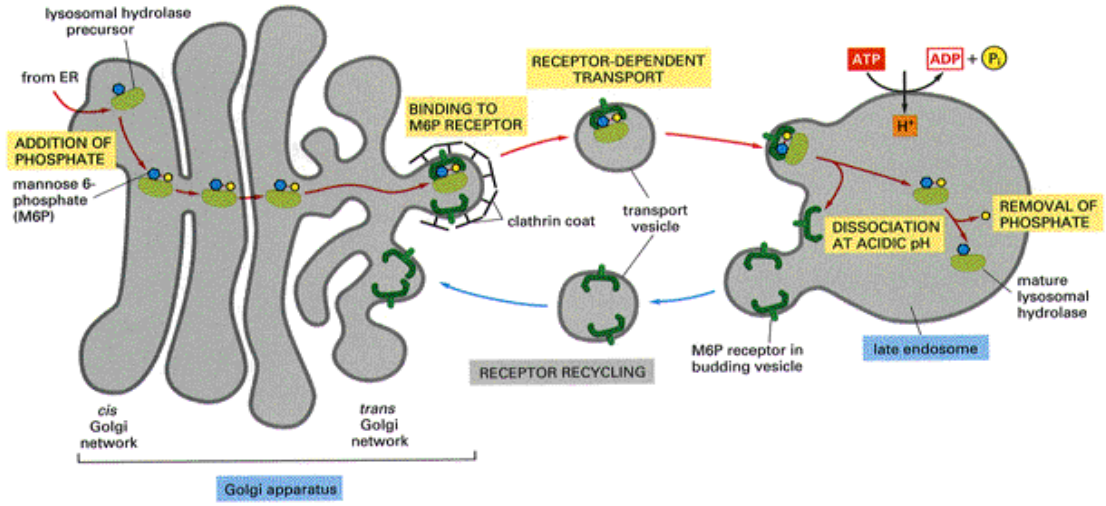

Fig. 8. Mechanism of lysosomal digestion
Nascent lysosomal enzymes (green balls) from the endoplasmic reticulum shuttle in the Golgi apparatus where they bind the mannose 6-phosphate receptor (M6PR). Most of the enzymes are then trafficked to the mature lysosome. [From The Art of MBoC[3], 1995]

Every moments several types of endogenous macromolecules, like proteins, neurotransmitter, signal transduction components, lipids, and/or cell ingested materials are delivered to the lysosome for organizing their degradation and their components recycling (Frandson et al., 2003), when admitted.
When this machinery is wrong and/or a genetic error occurs, cell integrity and viability are compromised, because an unbalance between anabolism and catabolism make collapse in physiological cellular homeostasis.

This elementary described situation is the theoretical basis of lysosomal storage diseases (LSDs) that are a group of genetic inherited disorders occurring in humans and animals when there is lowered activity of a lysosomal enzyme (Platt and Walkley, 2004). The result is the accumulation or storage of non-catabolised products, due to a defect in a hydrolytic enzyme, activator protein, transport protein, or enzyme required for the correct processing of other lysosomal proteins. This storage initiates a cascade of pathological dysfunction typically observed in the nervous system (Platt and Walkley, 2004).

The majority of LSDs have autosomal recessive inheritance and they are rare, occurring approximately 1 in 5000 to 8000 live births in the United States, Europe, and Australia (Meikle et al., 1999). Despite the rarity of these diseases, some of these disorders result in purely non-neurologic manifestations (e.g., Gaucher disease type 1), but many others are characterized by a wide range of neurologic symptoms, with or without somatic features, presenting from birth to adulthood and have devastating effects, imposing a significant burden on families and the community (Table 3). Still today, about 40 different genes have been identified as sites for mutations resulting in a LSD with neurologic involvement.

Disease	Defective Protein	Presenting Signs and Symptoms	Treatment Options†
GM1 gangliosidosis‡	Acid -galactosidase	IO: hypotonia, DD, coarse facial features HM, CRS (±) LO: DD, ataxia, dysarthria, PR, dystonia	SC,§ HSCT
GM2 gangliosidosis, B variant, Tay-Sachs disease‡	Hexosaminidase A	IO: hypotonia, hyperacusis, DD, CRS LO: ataxia, dystonia, psychoses, PR	SC, HSCT
GM2 gangliosidosis,O variant, Sandhoff disease‡	Hexosaminidase A & B	Similar to Tay-Sachs disease	SC
GM2 gangliosidosis, AB variant‡	GM2 activator protein	Similar to Tay-Sachs disease	SC
Fabry disease‡	α-Galactosidase	Acroparesthesia, pain crises, corneal opacities, fatigue,angiokeratomas	SC, ERT HSCT
Gaucher disease, types 2 and 3‡	Glucocerebrosidase	HSM, DD, strabismus, Sz, myoclonus, horizontal supranuclear gaze palsy	SC, ERT,HSCT
Niemann-Pick type A‡ F	Sphingomyelinase	HSM, hypotonia, DD, CRS (±)	SC
Niemann-Pick type C1‡	NPC1	Neonatal onset: jaundice, HSM, hypotonia LO: emotional lability, ataxia, dystonia, HSM (±), VSO	SC, HSCT
Niemann-Pick type C2‡	NPC2	Similar to Niemann-Pick type C1	SC
Metachromatic leukodystrophy‡	Arylsulfatase A	Late IO: weakness, hypotonia, DD, genu recurvatum JO: weakness, PR, ataxia, behavior changes AO: pyramidal or cerebellar signs, behavior changes, psychoses, dementia	SC, HSCT

Krabbe disease‡	Galactocerebrosidase	IO: spasticity, irritability, hypotonia, fisting, DD LO: spastic paraparesis, weakness, burning paresthesia, ataxia, weakness, vision loss	SC, HSCT
α-Mannosidosis‡	α-Mannosidase	DD, hearing loss, mildly coarse facial features (large jaw), mild DM	SC, HSCT
β-Mannosidosis‡	β-Mannosidase	DD, MR, hearing loss, mild facial coarsening, angiokeratomas	SC
Sialidosis, Mucolipidosis I‡	Sialidase	IO: NIFH, DD, coarse facial features, DM, HSM, PR,renal disease LO: myoclonus, CRS, ataxia, visual defects	SC
Sialic acid storage disease, Salla disease‡	Transport protein	IO: severe DD, fair hair and skin, HSM, coarse facial features LO: hypotonia, MR, ataxia, DD, speech delay, coarse facial features	SC
Galactosialidosis‡	Protective protein, cathepsin A	Neonatal onset: NIFH, HSM, severe DD IO and late IO: coarse facial features, HSM, kidney and heart defects, DD, DM, MR LO: coarse facial features, DM, corneal clouding, MR, ataxia,Sz, CRS(±)	SC
Fucosidosis‡	α-L-fucosidase	Spasticity, DD, coarse facial features, DM, MR, angiokeratomas	SC, HSCT
MPS I (Hurler and Hurler-Scheie)‡	α-L-iduronidase	Coarse facial features, DD, DM, MR, hearing loss, corneal clouding, hernias	SC, HSCT, ERT
MPS II (Hunter)	Iduronate-2-sulfatase	DD, DM, hearing loss, coarse facial features, joint stiffness	SC, HSCT, ERT
MPS III A (Sanfilippo)	Glucosamine-*N*-sulfatase	Aggressive behavior, DD, mildly coarse facial features, hirsute, coarse hair, mild DM	SC, HSCT
MPS III B‡	α-*N*-Ac-glucosaminidase	Similar to MPS III A	SC
MPS III C	AcCoA: α-glucosaminide-*N*acetyltransferase	Similar to MPS III A	SC
MPS III D	*N*-acetylglucosamine-6-sulfatase	Similar to MPS III A	SC

MPS VII‡	β-Glucuronidase	NIFH, DM, DD, coarse facial features, HSM, MR	SC, HSCT, ERT
Mucolipidosis II‡	UDP-N-Ac-glucosaminyl Phosphotransferase	Coarse facial features, DD, weakness, DM, gingival hyperplasia, macroglossia	SC, HSCT
Mucolipidosis IV	Mucolipin 1	DD, corneal opacities, retinal degeneration, strabismus	SC
Multiple sulfatase deficiency‡	Sulfatase modifier protein	DD, ichthyosis, coarse facial features, deafness, mild DM, PR	SC
Neuronal Ceroid Lipofuscinosis 1	Palmitoyl-protein thioesterase 1	Vision loss, motor dysfunction, hypotonia, DD, MR	SC, HSCT
Neuronal Ceroid Lipofuscinosis 2	Tripeptidyl peptidase 1	Sz, motor dysfunction, DD, MR, ataxia, dementia	SC
Saposin defect, MLD type‡	Saposin B	Similar to JO MLD	SC
Saposin defect, Gaucher type‡	Saposin C	Similar to Gaucher disease, type 3	SC
Saposin defect, generalized type‡	Prosaposin	HSM, DD, motor abnormalities, exaggerated Moro reflex, MR	SC
Aspartylglucosaminuria	Aspartylglucosaminidase	Delayed speech, motor clumsiness, mildly coarse facial features, behavioral problems	SC
Farber disease‡	Acid ceramidase	Painful and swollen joints, nodules, DD, hoarse cry, hypotonia	SC, HSCT
Wolman disease‡	Acid lipase	HSM, vomiting, diarrhea, anemia, PR	SC, HSCT
Schindler disease	α-N-acetylgalactosaminidase	DD, blindness, Sz, spasticity, PR	SC
Pompe disease	α-Glucosidase	Hypotonia, DD, cardiac enlargement	SC, ERT

Table 3. Presenting features in Lysosomal Storage Diseases with prominent neurologic findings
Abbreviations: AO, adult onset; CRS, cherry-red spots; DD, developmental delay; DM, dysostosis multiplex; ERT, enzyme replacement therapy; HSCT, hematopoietic stem cell transplantation; HM, hepatomegaly; HSM, hepatosplenomegaly; IO, infantile onset; JO, juvenile onset; LO, late onset; L, leukocytes; MLD, metachromatic leukodystrophy; MPS, mucopolysaccharidosis; MR, mental retardation; NIFH, nonimmune fetal hydrops; PR, psychomotor regression; Sz, seizures; SC, supportive care; VSO, vertical supranuclear ophthalmoplegia. †HSCT is not available for all patients with a given diagnosis, and ERT may be in use or only at the stage of preclinical trials. The use of these treatments in a few patients does not necessarily indicate a successful outcome.‡Diseases diagnosed in author's laboratory.§SC indicates any procedure performed to alleviate pain, discomfort, and seizures and may include splenectomy and kidney transplantation when indicated.
[Adapted from Wenger et al., 2003]

The differences in symptoms demonstrate that even if most lysosomal enzymes are ubiquitous, the effect of undigested-substrate accumulation depends on the cell or tissue type in which the substrate is synthesized with a high turnover. For example, accumulation of glycogen in Pompe involves myopathy for its role in muscle metabolism, while several sphingolipidosis are characterized by severe neuropathology, because of their high concentration in the brain.

Thus, owing to the complexity of the storage products (Table 4) and differences in their tissue distribution and rates of accumulation, the disease can cause pathologic changes in multiple organ systems or can be confined to the nervous system (Wenger et al, 2003).

Classification	Accumulated Substrate	Disease Examples	Species
Lipidoses	Lipids. Includes sphingolipids, cholesterol esters and triglycerides	Krabbe disease	Humans, mice, dogs, sheep, monkey
		Gaucher disease	Humans, mice, dogs, sheep
		Wolman disease	Humans, mice
Mucopolysaccharidoses (MPS)	Mucopolysaccharides (glycoaminoglycans)	MPS I	Humans, mice, cats, dogs
		MPS II	Humans, mice, dogs
		MPS III	Humans, dogs, cattle
Glycogenosis	Glycogen	Pompe disease	Humans, mice, cattle, dogs, cats
Glycoproteinoses	Glycoproteins and/or oligosaccharides	Fucosidosis	Humans, dogs
		α- mannosidoses	Humans, cattle, mice, guinea pig, cats
		Sialidosis	Humans, mice

Table 4 Categories of LSDs based on accumulated substrate
[Modified from Hopwood et al., 2004]

The treatment options for LSDs, like enzyme replacement therapy, hematopoietic stem cell transplantation are not completely effective; so the exit is unfavorable. Supportive care became necessary and a definitive protocol of treatment remains limited despite ongoing research (Kolter and Sandhoff, 2006; Platt and Walkley, 2004; Wenger et al., 2001).

Therefore, it is critical that the patients be diagnosed as early as possible, by recognizing the symptoms from the beginning. Several structures perform newborn screening for LSDs to identify presymptomatic individuals that may be candidates for early therapeutic intervention. Bio-banks institution could be important in the next future, due to the augmented efficiency of scientific searching methods.

Krabbe disease description

Krabbe disease (OMIM #245200, OMIA #1140/000578) is one of the classic genetic LSDs with autosomal recessive inheritance that affects both CNS and PNS in several species including humans, rhesus macaques, dogs, mice (Suzuki and Suzuki, 1985; Suzuki, 2003) and sheep (Pritchard et al., 1980). In 1916, the Danish neurologist Knud Haraldsen Krabbe described the presence of globoid cells in the brain (Krabbe, 1916). So the disease was named after him and later globoid cell leukodystrophy, due to the presence of multinuclear (globoid) macrophages in the white matter. This was the unique microscopic pathological characteristic of Krabbe disease and reliable diagnostic method until the beginning of the 70s, when Suzuki and his colleagues (Suzuki et al., 1970a; Suzuki et al., 1971) identified the genetic deficiency of enzyme galactosylceramidase (galactocerebroside β-galactosidase) (GALC) as the cause of human and canine globoid cell leukodystrophy.

GALC is a lysosomal enzyme that catalyzes the degradation of galactose from galactosylceramide (Gal-cer) and galactosylsphingosine (psychosine, Psy). It acts in myelin turnover to catabolize the myelin lipid (Lefebvre and Vartanian, 2002; Wenger et al., 2001). In the normal nervous system, these substances are processed by the lysosome (Kolter and Sandhoff, 2006) and recycled components are able to enter the remyelination pathway (Lefebvre and Vartnanian, 2002; Suzuki, 2003). Occurring mutations in the GALC gene (Luzi et al., 1997; Rafi et al., 1995; Sakai et al., 1996; Victoria et al., 1996) result in a lower (Wenger et al., 2001) or loss activity of GALC and lipid degradation during myelin turnover is impaired (Suzuki, 2003; Wenger et al., 2001). Remyelination does not occur effectively in Krabbe disease (Suzuki, 2003; Wenger et al., 2001), but myelin turnover continues (Suzuki, 2003).

For this reasons, Krabbe disease is a sphingolipidosis with the peculiarity that, despite the GALC deficiency, galactosyceramide does not increase in the brain of patients. However, psychosine accumulates in the brain causing death of myelin-generating cells.

The galactosylceramidase deficiency results in degeneration of oligodendrocyte, astrocytic gliosis, and appearence of globoid cells, and demyelination that. Alteration of neuronal conduction and neurological dysfunctions are hallmark of pathology.

Krabbe disease in humans

Krabbe disease in humans is typically a neurodegenerative disease of infancy, but there are rare examples in which Krabbe disease has been diagnosed in older children and adults (Wenger et al., 2001).

Symptoms of Krabbe disease usually indicate CNS and PNS involvement, particularly the cerebellum (Jacob et al., 1973; Suzuki, 2003) which controls the refinement and execution of movement and the co-ordination of muscle activity (Starr and Taggart, 2001), as region of the brain responsible for the initiation of movement and conscious sensation. Clinical sign of disease progress is the loss of higher cerebral functions (Suzuki, 2003; Wenger et al., 2001).

The symptoms of the infantile form (95% of known cases) usually onset at 3-6 months and initially are characterized by generalized hyperirritability, hypersensitive to the external environment, stiffness of the limbs (Suzuki and Suzuki, 1985; Suzuki, 2003; Wenger et al., 2001), and episodic fever of unknown origin. The cerebrospinal fluid protein level is already highly elevated and rapid and severe motor and mental deterioration develops: psychomotor functions deteriorate with marked hypertonicity, extended and crossed legs, flexed arms, and the backward-bent head. At the end stage of the disease, sometimes reached within a few weeks or months, the infant is decerebrate and blind and become unresponsive and unaware of their surroundings. In this case, rarely patients survive beyond two years of age (Suzuki, 2003; Wenger et al., 2001) and often die of respiratory infections.The parents of an affected child are obligate heterozygotes and therefore each parent carries one normal and one mutated *GALC* allele, the measured GALC enzyme activity can range widely in carriers because of polymorphisms in the normal copy of the gene. Although some parents have quite low GALC enzyme activity measured *in vitro*, none has clinical disease. At conception, each sib of an affected individual has a 25% chance of being affected, a 50% chance of being an asymptomatic carrier, and a 25% chance of being unaffected and not a carrier. Once an at-risk sib is known to be unaffected, the chance of his/her being a carrier is 2/3.

The clinical phenotype of the later-onset forms is much more variable and it is often divided into two groups: the late infantile (onset 6 months to 3 years), and the juvenile (onset 3-8 years). In the late infantile group, irritability, psychomotor regression, stiffness, ataxia, and loss of vision are frequent initial symptoms. The course is progressive, resulting in death in approximately 2 to 3 years after the onset. In the juvenile group, patients commonly develop loss of vision, together with hemiparesis, ataxia, and psychomotor regression.
Sometimes adult patients have a milder phenotype and a slower rate of progression. Some adult patients could have a normal life span. While some individuals remain stable for long periods of life, others show a steady decline in vegetative state and death. The cerebrospinal fluid protein is normal or only mildly elevated in juvenile or adult patients.

The offspring of an individual with adult-onset Krabbe disease is obligate heterozygotes (carriers) for a disease-causing mutation in *GALC*. Aunts and uncles, and each grandparent is at a 50% risk of being a carrier.

The diagnosis of Krabbe disease is currently made by a combination of methods including histopathologic examination, enzymic assay and genetic testing (Suzuki, 2003; Wenger et al., 2001). Historically the disease was diagnosed *post mortem* by histopathologic observations of central nervous tissue (Krabbe, 1916; Jacob et al., 1973; Schochet et al., 1976). This method continues to be used in conjunction with an enzyme assay to confirm diagnosis (Suzuki, 2003; Wenger et al., 2001). This enzyme assay was developed in the 1970s (Miyatake and Suzuki, 1972a; Suzuki and Suzuki, 1970) and measures the activity level of galactosylceramidase (Miyatake and Suzuki, 1972a). The activity of galactosylceramidase is used to determine the phenotype of an individual as affected or normal. While the enzyme assay is effective in diagnosing Krabbe disease, it cannot be used to conclusively identify carriers (Wenger et al., 2001). Recently, the advent of molecular technology has allowed for mutations causing the deficiency of galactoslyceramidase to be identified (Fu et al., 1999; Rafi et al., 1995). The identification of several mutations has facilitated the confirmation of Krabbe disease diagnosis through the development of PCR-based tests designed to check for the presence these mutations (Fu et al., 1999; Rafi et al., 1995; Wenger et al., 2001). Genetic testing is a particularly useful diagnostic tool in families where parents are known carriers. In this situation, PCR-based tests are employed in prenatal diagnosis using a sample of amniotic or chorionic villus cells (Wenger et al., 1997).

More recently, a new method for a rapid diagnosis is undergoing investigation: the quantification of psychosine, the toxic metabolite of Krabbe disease, in biological sample by using electrospray ionization-tandem mass spectrometry (Whitfield et al., 2001, Zanfini et al., 2013).

The major histopathological changes are extensive demyelination, gliosis and presence of globoid cells in the white matter (Fig. 9).

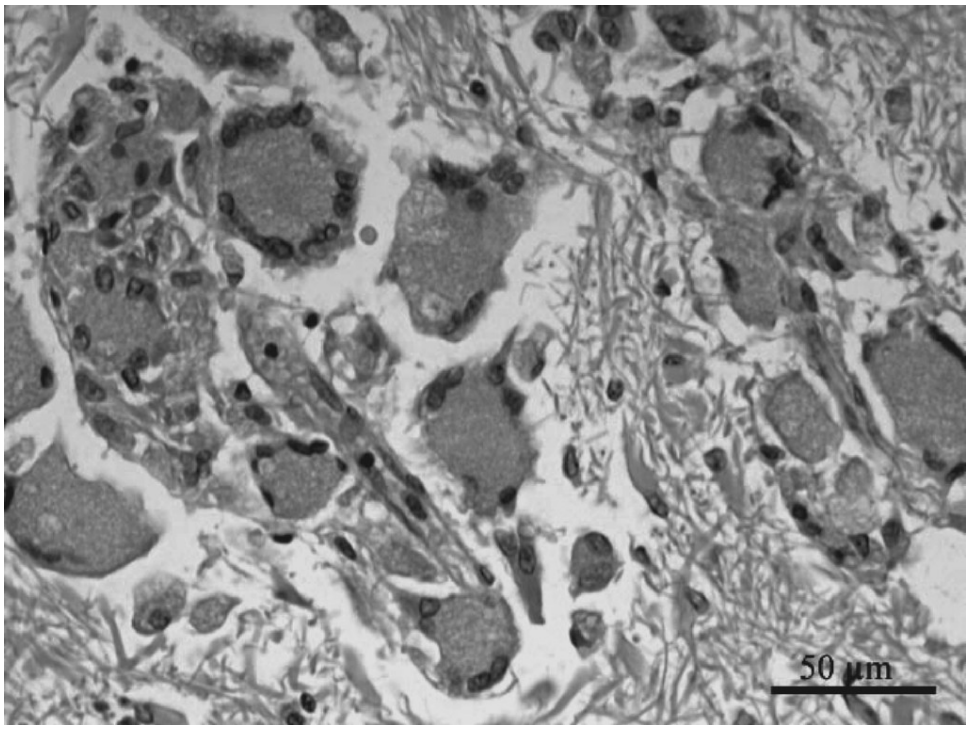

Fig. 9 Typical histology of the white matter of a patient with infantile Krabbe disease Hematoxylin-eosin stain. The cellular architecture is highly disrupted with nearly complete loss of myelin and the oligodendrocytes, which are replaced by severe astrocytic gliosis and many characteristic multinucleated macrophages ("globoid cells").[From Matsuda and Suzuki]

Loss of oligodendrocyte and myelin is massive at the late stage of the disease and astrocytic gliosis replaces this loss. In the areas of active demyelination, multinucleated macrophages, the globoid cells, are often clustered around blood vessels. The globoid cells contain inclusions that are moderately electron-dense. These inclusions are periodic acid-Schiff (PAS) positive (Yunis et al., 1970). Both mononuclear and multinucleated globoid cells are similar ultrastructurally, except for the number of nuclei.

The peripheral nerves tend to be firm and abnormally enlarged and white on gross inspection. The major pathological features are marked endoneurial fibrosis, proliferation of fibroblasts, segmental demyelination, and infiltration of perivascular aggregation of histiocytes/macrofages containing PAS positive materials similar to those in globoid cells in the brain. Thinly myelinated fibers suggestive of remyelination may be present. Axonal degeneration of varying degrees has been reported.

Human GALC: gene and protein

The galactosylceramidase gene, GALC (GenBank database accession no. 119970) was cloned in 1993-1994 (Chen et al. 1993b; Sakai et al. 1994). GALC gene has been mapped to human chromosome 14 by linkage analysis and to the region 14q13 by in situ hybridization using a probe from the cDNA sequence.

The full-length complementary DNA consists of 3795 bp, including 2007 bp of the coding region, 47 bp of 5' untranslated sequence, and 1741 bp of 3' untranslated sequence. The base and amino acid sequences show no similarities to and thus no suggestion of evolutionary relationship with other β-galactosidases or any other known genes. The organization of the human gene was characterized in 1995 (Luzi et al. 1995). It consists of 17 exons and 16 introns spread over about 58 kb. Other than the first and last exons, the other exons are relatively small ranging from 39 to 181 bp. The introns range in size from 247 bp for intron 2 to about 12 kb for intron 10.

Mutation analysis of the human GALC gene was facilitated by the cloning and sequencing of GALC cDNA (Chen et al., 1993; Rafi et al., 1995; Sakai et al., 1994). This allowed DNA obtained from Krabbe affected individuals to be sequenced and analyzed against the normal GALC gene. Over 70 disease-causing missense, nonsense mutations, deletions, and insertions have been identified in the human galactosylceramidase gene (Fig.10) (Wenger et al. 2001).

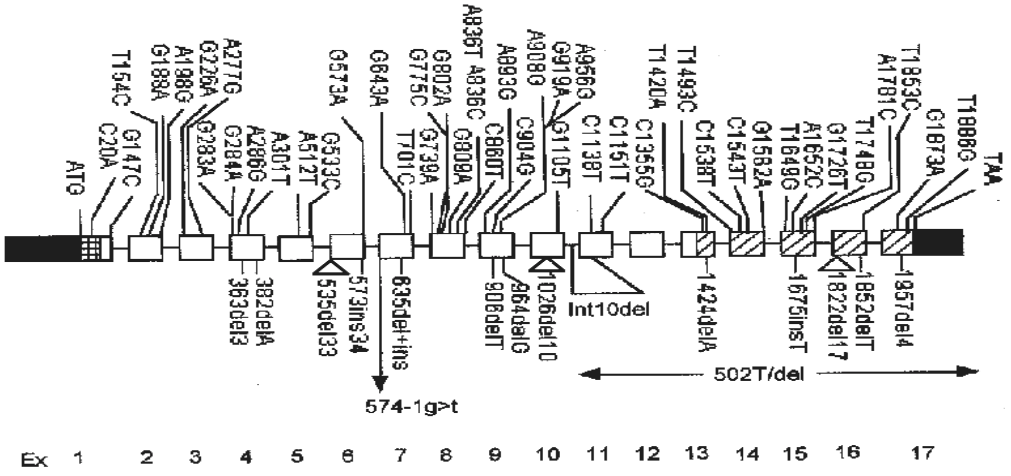

Fig. 10. Schematic drawing of the GALC gene with each box representing an exon
The black area represents untranslated regions, the open boxes contain the coding region for the 50- to 53-kDa subunits, and the cross-hatched boxes contain the coding region for the 30-kDa subunit. The numbers refer to the location of the mutation in the cDNA sequence counting from the A of the initiation codon. Missense mutations are shown above the gene and deletions and insertions are shown below the gene.
[From Wenger at al., 2000]

Found in 40-45% of the disease-causing alleles in patients with infantile form and common in North Europe and the United States, including those with Mexican ancestry, the 30 kb deletion is associated with a C to T transversion at cDNA position 502 (Rafi et al., 1995; Wenger et al., 1997). The C to T transversion was a polymorphism that does not affect galactosylceramidase activity, but eliminates the entire coding region for the 30 kDa subunit and about 15% of the coding region for the 50 to 52 kDa subunit. This mutation probably originated in Sweden, it is spread throughout Europe, and it is found in patients from India.

In the Japanese population, the most common mutation is a 12 bp deletion with a 3 bp insertion. This results in the deletion of 5 amino acids and the insertion of 2 amino acids which impacts on the quaternary structure of galactosylceramidase (Tatsumi et al., 1995; Wenger et al., 1997).

Many patients with late-onset form of Krabbe disease have one copy of the G809A (G270A) mutation. This missense mutation results in the production of a small amount of active enzyme that delays the onset of the disease.

Other genetic and/or environmental factors are involved in the expression of the disease. Many patients are compound heterozygotes having different mutations in the two copies of the GALC gene. This makes difficult genotype-phenotype correlations. It is clear from mutation analysis that both 30 kDa and 50 kDa subunits must be functional to have active enzyme. Moreover, it is clear that phenotype prediction cannot be made on the nature or location of missense mutations. Most patients are compound heterozygotes.
The large number of mutations means that there are also a large number of possible genotypes. This means that compound heterozygotes may occur (Wenger et al., 1997). Compound heterozygotes are individuals that have two different mutated alleles at the same locus. In many cases where compound heterozygotes are identified, they have low to intermediate galactoslyceramidase activities. In these cases, clinical signs of Krabbe disease may be not present or atypical (Wenger et al., 1997). This has made difficult to correlate phenotype with genotype in human Krabbe disease (Fu et al., 1999; Wenger et al., 1997; Wenger et al., 2000).
Table 5 shows the common *GALC* polymorphisms and mutations, even if, three years ago, 15 new GALC gene mutations causing Krabbe disease were identified (Tapino et al., 2010).

Class of Variant Allele	DNA Nucleotide Change	Protein Amino Acid Change	% of All Alleles	Reference Sequences
Normal (benign polymorphisms)	c.550C>T (502C>T)	p.Arg1 84Cys (Arg1 68Cys)	4%-5%	NM_0001 53 .3 NP_0001 44 .2
	c.742G>A (694G>A)	p.A sp248A sn (A sp232A sn)	8%-1 0%	
	c.1685T>C (1637 T>C)	p.Ile562Thr (Ile546Thr)	35%-45%	
	c.[550C>T;1 685T>C] (502C>T + 1 637 T>C)	p.[Arg1 84Cys;Ile562Thr] (Arg168Cys + Ile546Thr)	<2%	
Pathologic	(30-kb deletion)	--	40%-50%	
	c.1586C>T (1538C>T)	p.Thr529Met (Thr51 3Met)	5%-8%	
	c.1700A >C (1 652A >C)	p.Ty r567 Ser (Ty r551 Ser)	5%-8%	
	c.1472delA (1 424delA)	p.Lys491Argfs*	2%-5%	
	c.857 G>A (809G>A)	p.Gly 286A sp (Gly 27 0A sp)	1 %-2%	

Table 5. Common GALC Polymorphisms and Mutations
[From Krabbe Disease - GeneReviews™ NCBI Bookshelf by Wenger, 2011]

Recently, an interesting work showed that a mutation with high rate of late onset forms. The c.121G>A (p.Gly41Ser) mutation gives a longer survival phenotype. It seems to be detected only in a restricted population coming from the same demographic area, the north of the city of Catania, Sicilia (Fiumara et al, 2011).

There is a relatively broad range of galactosylceramidase activities in the "normal" population and among obligate heterozygotes. This makes enzyme activity-based carrier testing in the general population nearly impossible. These phenomena can be explained at least, partially by the presence of polymorphisms in the galactosylceramidase gene that results in amino-acid substitutions. Some polymorphisms generate galactosylceramidase proteins that are enzymatically less active. Polymorphisms can also play a role in the development of clinical disease when inherited either in multiple copies, on the same allele with another mutation, or together with a known disease-causing mutation on the other chromosome (Luzi et al. 1996).

The *GALC* encoded-protein consists of 669 amino acids with six potential glycosylation sites. The first 26 amino acids have the characteristic of a leader sequence. The precursor protein is approximately 80 to 85 kDa; the active form consists of two subunits, a 50 to 52 kDa and a 30 kDa mature forms, (Chen et al., 1993a), obtained by the action of proteases probably localized in lysosomes. The active GALC is a hydrolytic enzyme with an acid pH optimum localized in the lysosome. The enzyme is specific for certain glycolipids with a terminal galactose moiety in the β–anomeric configuration. The major natural substrate is galactosylceramide, which is almost exclusively localized in the myelin sheath. The galactosylceramide is a sphingolipid containing sphingosine, galactose and fatty acid. *In vivo* degradation of the substrates requires an activator protein, saposin A, in addition to the enzyme galactosylceramidase. In fact, the mouse model with a point mutation in the saposin A region shows pathological features resembling late-onset form of Krabbe disease (Matsuda et al. 2001). The first report of a saposin A mutation in humans leading to pathological consequences: a six month infant girl, originally diagnosed as Krabbe disease patient, was later found to have a deficiency of the saposin A protein (Spiegel et al. 2005).
Other known natural substrates are psychosine, monogalactosyldiglyceride, and the precursor of seminolipid (1-alkyl, 2-acyl- 3-galactosyl glycerol). The metabolism of galactosylceramide and psychosine is important to understand the pathology of this disorder (Fig. 11).

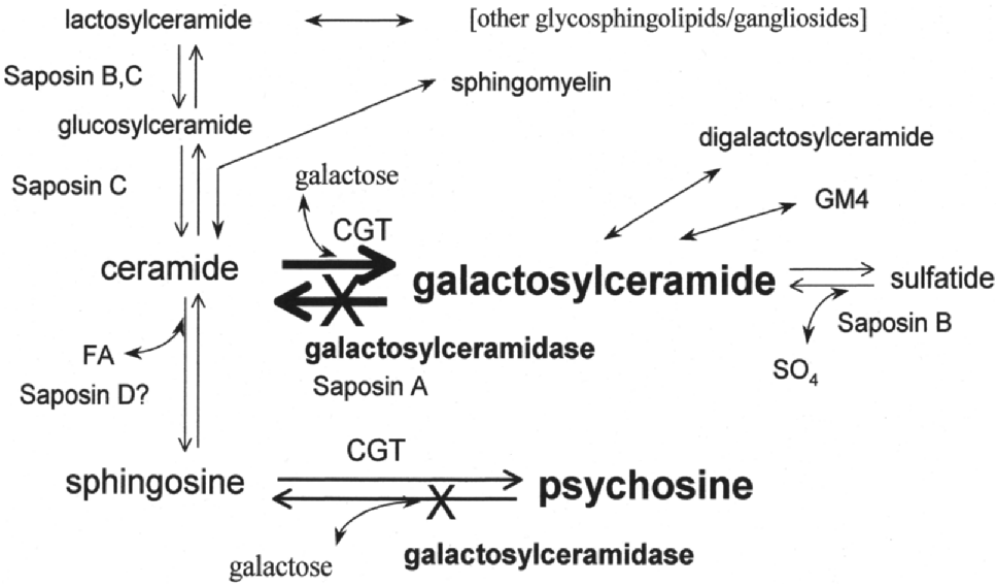

Fig. 11. Metabolic pathways pertinent to galactosylceramide and related compounds
In the synthetic pathway, sphingosine is first acylated to ceramide, which in turn is galactosylated by UDPgalactose:ceramide galactosyltransferase (CGT) to form galactosylceramide. The same enzyme can galactosylate sphingosine directly to generate psychosine. Both galactosylceramide and psychosine are degraded by galactosylceramidase, which is genetically deficient in Krabbe disease. *In vivo* degradation of galactosylceramide requires, in addition to the enzyme, a sphingolipid activator protein, saposin A. Galactosylceramide is further sulfated to form sulfatide. Both galactosylceramide and sulfatide are characteristic myelin glycolipids**.**

The characteristic biochemical feature of Krabbe disease is the lack of accumulation of the undegraded galactosylceramide in the brain, explained by the early degeneration of the myelin forming cells and the block in the synthesis of galactosylceramide (Vanier et al., 1975; Svennerholm et al. 1980). However, as related toxic metabolite (Taketomi et al., 1964), psychosine (galactosylsphingosine) accumulates abnormally and is considered the key compound in the pathogenesis of the disease (Miyatake and Suzuki, 1972; Vanier and Svennerholm, 1976; Suzuki, 2003). It is a minor compound in normal brain, but is present in relatively large amounts in brain white matter, peripheral nerves of humans, and animal models with GALC deficiency. GALC is the only enzyme capable of hydrolyzing psychosine (Fig.11).

Recently, the crystal structures of GALC enzyme and the GALC-product complex was obtained, revealing novel domain architecture with a previously uncharacterized lectin-domain did not observe in other hydrolases (Fig.12). All three domains of GALC contribute residues to the substrate-binding pocket, and disease-causing mutations are widely distributed throughout the protein (Deane et al., 2011).

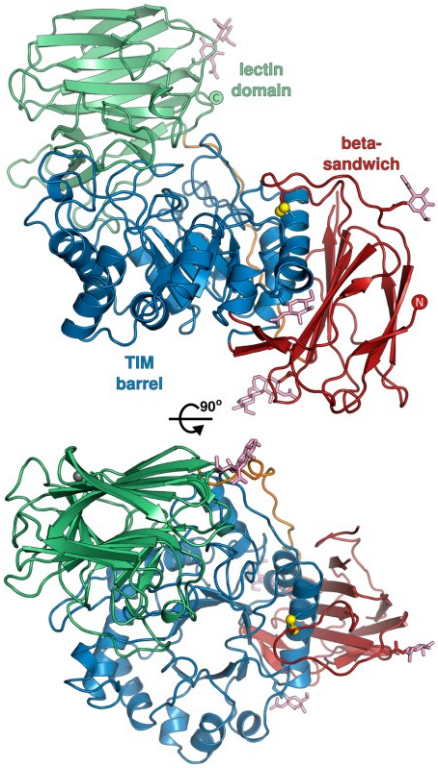

Fig. 12. Structure of GALC shown in two orthogonal views
Colors: β-sandwich (red), TIM barrel (blue), linker (orange), and lectin domain (green); disulfide bond (yellow spheres), calcium ion (gray sphere), and glycosylation moieties (violet sticks) The N and C termini are marked by labeled circles (Top).[From Deane at al., 2011]

These structures, normal and mutated, could be useful for computational analyses, as docking, because they provide the atomic framework for drugs design and molecular interaction.

Animal models

Animal models (Fig. 13) are valuable resources where investigation in human subjects may be limited by ethical concerns or limited availability. Since Krabbe disease primarily affects children, ethical concerns of disease investigation in humans can be manifold (Platt and Walkley, 2004; Suzuki and Suzuki, 1985). There is also the rarity and variability of the disease that challenges experimental design and analysis (Wenger, 2000). Thus, animal models can overcome both these difficulties, providing test subjects that can be used in studies to increase understanding and knowledge of the disease, as well as in treatment trials to improve the quality of life for affected individuals (Wenger, 2000). Krabbe disease has different naturally occurring animal models: the rhesus monkey (Baskin et al., 1998; Borda et al., 2008), the sheep (Pritchard et al., 1980), the mouse (Duchen et al., 1980) and the dog (McGraw and Carmichael, 2006; Wenger et al., 1999).

Fig. 13. Mouse, West Highland white terrier and rhesus monkey Krabbe disease affected
[From Wenger, 2010]

All these models have been well characterized and all mutations identified. The clinical course, analytical biochemistry, severity of pathological features, molecular defects and potential use as a model to attempt therapeutics trials are basically similar in all species and have been described in detail (Suzuki et al, 1985, Wenger et al., 2000a). Of these models, the mouse model, known as the *twitcher* mouse, has been studied in depth and a new transgenic mouse has been developed (Luzi et al., 2001). As it has been demonstrated in human Krabbe disease, the animal models have progressive increase of psychosine in nervous tissues, and mice have a large increase of in galactosylceramide in kidney and galactosyl-alkyl-acyl-glycerol (GalAAG) in the testis (Matsuda et al., 2001, Luddi et al., 2005). Fletcher (Fletcher et al., 1966) as a model of a lysosomal disease first described the dog model West Highland white terrier. The clinical course of the dog is often slower and milder than the human, but the histopathological features are identical to the human disease. Suzuki has confirmed the enzymatic defect (Suzuki et al., 1970c) and Victoria and colleagues have cloned the cDNA of the canine GALC and identified the causing mutation. Two nucleotide changes were found in affected dogs, an A to C at cDNA position 473 (Y158S) and a C to T transition at position 1915 (P639S) (Victoria et al., 1996). The coding sequence cDNA of the canine GALC is long as the human

cDNA and compared to the human sequences, the nucleotide and protein sequence have homology of 88% and 90%, respectively.

The *twitcher* mouse (Fig. 14), the mouse model of the GLD, has been discovered in the Jackson laboratories in 1980 (Kobayashi et al., 1980).

Fig. 14. The normal (top) vs twitcher mouse (bottom)
Lose weight and a hunched back are characteristic of twitcher mouse.
[From De Gasperi et al., 2004]

The *twitcher* mouse is the only natural mouse model of a sphingolipidosis (Suzuki et al., 1983). The single base substitution for a nonsense mutation at codon 339 (TGG>TGA) is inherited as an autosomal recessive transmission. The mapping of the mouse mutation on chromosome 12 was very useful in mapping the human disease to human chromosome 14. The neuropathological and ultrastructural features of abnormal inclusions in globoid cells and glial cells in this mutant mouse were almost identical to those in human as well in canine. However, in contrast to human GLD, in which the CNS is severely damaged, peripheral nerves tend to be involved more severely in the murine mutant. The presence of the typical cytoplasmic inclusions in the kidney and lymph nodes, together with significant increased amount of galactosylceramide in the kidney of the mutant, is another difference between human and murine GLD affected. These differences may be considered as a species difference (Takahashi et al. 1983). In *twitcher* mouse, neurological symptoms such as twitching and hind leg weakness occur at approximately 20 postnatal days. They deteriorate neurologically and lose weight, around 30 days old. The symptoms progress with paralysis of hind limbs and, ultimately, the death occurs at about 40 postnatal days.

Therapy

In symptomatic infantile patients, treatment has been limited to a supportive symptomatic care, while in patients with the late onset form of the disease, allogeneic bone marrow transplantation (BMT) has been reported to be beneficial. Following the engraftment, an arrest in the progression and a significant improvement in the clinical course of the disease was observed. The survival data show a significant advantage of the engrafted compared to non-transplanted patients (McGowan et al., 2000). The MRI, as well as spectroscopic examination of the brain, showed further evidence that positive changes occurred in the central nervous system following long-term engraft, providing a better quality of life (Loes et al., 1999; McGowan et al., 2000; Krivit et al., 1999a, b). In utero BMT has been attempted with little success (Blakemore et al., 2004).

Escolar and colleagues (Escolar et al., 2005) reported some success in treating pre-symptomatic infantile patients with Krabbe disease during the neonatal period using umbilical cord blood. While many of these children have recovered well cognitively, many have significant motor deficits. In older symptomatic infants (4-11 months) umbilical cord blood transplantation stabilized the disease progression, but the neurological impairments were not reversed. Umbilical-cord blood transplantation helps to overcome the lack of a matched donor and to reduce the time needed to find an unrelated compatible donor. In addition, umbilical-cord blood is likely to contain a larger number of uncommitted stem cells that can help regeneration and repair of the nervous tissue. While the long-term prognosis is not known, there is clear evidence that transplantation of pre-symptomatic infantile patients with hematopoietic stem cells (HSC) from umbilical cord blood slow the clinical course of the disease, and early death of untreated siblings. Although it is too early to reach a conclusion, hematopoietic stem cells and umbilical cord blood transplantation can be seriously considered in pre-symptomatic infantile cases of Krabbe disease.

Bone marrow transplantation (BMT) has been performed many times in the *twitcher* mouse. This treatment extends the life of the affected mice to about 100 days compared to 40 days of the untreated mice (McGowan et al., 2000; Krivit et al., 1998). In the treated mice, there was generalized significant elevation in the GALC activity but little evidence for remyelination, and while there was less psychosine accumulated, the brain pathology was identical to that found in the untreated mice (Ichioka et al., 1987). The rational for using BMT to treat neurologic disorders and Krabbe disease is the replacement of brain microglial cells by marrow-derived macrophages. In theory, these cells can secrete the missing protein (in this case GALC) and this can be taken up by the neighboring cells, such as oligodendrocytes, and correct their enzymatic defect.

In vitro correction of the enzyme deficiency utilizing retroviral vectors containing the GALC cDNA were performed in fibroblasts, glial cells, astrocytes, and oligodendrocyte from *twitcher* mouse (Gama Sosa et al., 1996; Rafi et al.,1996; Torchiana et al., 1998; Costantino-Ceccarini et al., 1999; Luddi et al., 2001). These studies demonstrated that oligodendrocytes from *twitcher* mouse can be biochemically and phenotypically corrected *in vitro* utilizing retrovirally mediated gene transfer as well as enzyme uptake (Costantino-Ceccarini et al., 1999; Luddi et al., 2001).

Injection of recombinant viral vectors or transplantation of enzyme producing cells has been used to target the therapeutic enzyme to the brain. The results of the experiments using adeno-associated virus (AAV) in the *twitcher* mice showed improvements such as prolonged life span, reduced psychosine levels, increased body weight and better performance in behavioral tests, but they die with symptoms similar to those of the untreated mice (Shen et al., 2001; Rafi et al., 2005; Lin et al., 2005). Additional strategies are need to develop more efficient CNS-directed gene therapy.

Another therapeutic intervention has been explored in *twitcher* mice: the substrate reduction therapy. Instead of enhancing enzymatic activity, the objective of substrate reduction therapy is to slow the synthetic rate of the accumulating glycolipids. A reduction in the synthesis of substrates should enable a residual degradation capacity of the defective enzyme to keep up with a slower accumulation of substrates. Substrate reduction therapy using L-cycloserine, an inhibitor of 3-ketodihydrosphingosine synthase, has been performed in wild type and in *twitcher* mice. In wild type mice, administration of L-cycloserine (Sundaram et al., 1984, Sundaram et al., 1989) lowered the levels of sphingolipids including galactosylceramide within myelin membrane (Miller et al., 1998). In *twitcher* mice, L-cycloserine treatment prolonged the lifespan of about 31%, and it delayed the onset of clinical symptoms and attenuated pathological signs (LeVine et al., 2000). This treatment augmented the beneficial effects on lifespan when used in conjunction with BMT (Biswas et al., 2002). Substrate reduction therapy, has also been tested in *twitcher* mice on the B6:CAST/Ei background. This treatment increased the lifespan and reduced the psychosine levels of the affected mice by 45% (Biswas et al., 2003).The evaluation of dietary was investigate in *twitcher* mice: an increased life span and the weight gain were observed with a galactose free diet enriched in soy isoflavones and in a pool of antioxidants molecules, such as L-glutathione, coenzyme Q10, xanthophylls. In addition, the antioxidant treatment is reported to suppress the apoptotic effects of psychosine in cultured oligodendrocyte (Pannuzzo et al., 2010) Treatments with chemical chaperones are under investigation,also (Parenti G., 2009).

However, currently there is no definite cure for Krabbe disease.

MECHANISM OF KRABBE DISEASE PATHOGENESIS

The biochemical of GALC enzyme and the related lipids should be kept in mind for understanding and try to explain the clinical hallmarks of Krabbe disease and the histopathological and biochemical features (Fig. 15). Since the underlying genetic malfunction in the production of galactosylceramidase is described, the enzyme substrates were investigated.

Essentially the deficiency of this enzyme results in the impaired degradation of two lipids: galactosylceramide, the primary substrate of GALC, and psychosine, its secondary substrate (Suzuki, 2003).

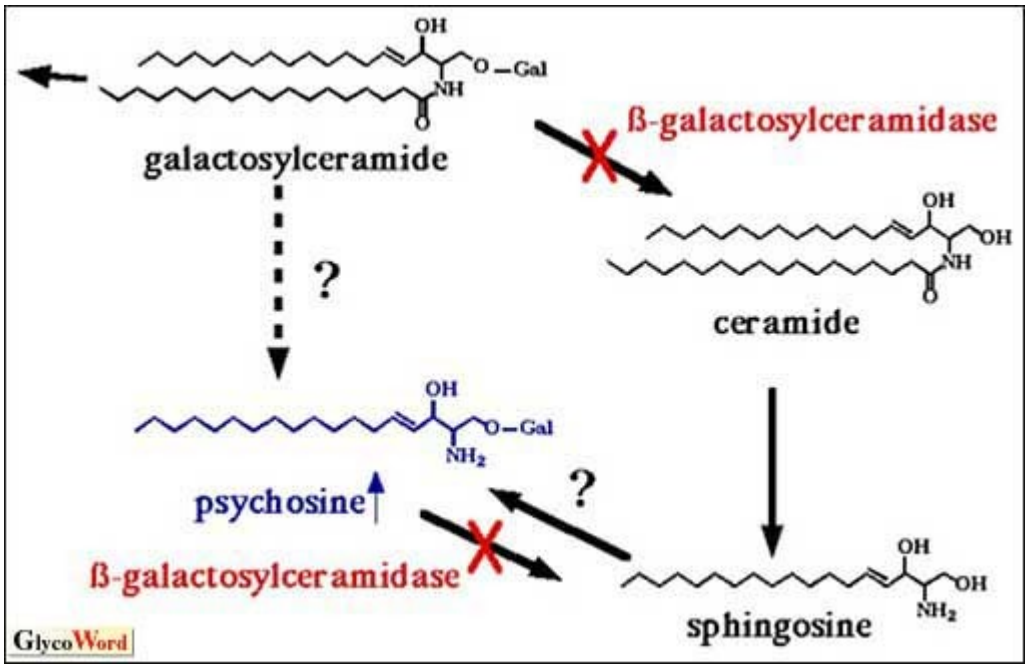

Fig. 15. Metabolic pathway of GalCer and biosynthesis pathway of Psy in GLD
[From Kanazawa, 2002 in Glicoword index]

Unlike most other LSDs, the primary substrate is not found at elevated levels in the nervous tissue of Krabbe affected individuals (Suzuki, 2003).
On the contrary, psychosine has been found at high concentrations and is believed to cause the pathological features of the disease (Igisu and Suzuki, 1984; Kobayashi et al., 1980; Svennerholm et al., 1980). This is known as the 'psychosine hypothesis' (Miyatake and Suzuki, 1972a).

However, both substrates cooperate for instauration of typical signs of the disease, acting with two separate but related pathogenetic mechanisms.

Galactosylceramide

Galactosylceramide (Galβ1-1'Cer) is the principal glycosphingolipid in brain tissue, hence the trivial name "cerebroside", which was first conferred on it in 1874. Galactosylceramides are found in all nervous tissues, and they can amount to 2% of the dry weight of gray matter and 12% of white matter. They are major constituents of oligodendrocytes in the CNS and of Schwann cells in the PNS (Lefebvre and Vartnanian, 2002). Therefore, galactosylceramide and its sulfatide derivates are characteristically the glycolipids of the myelin sheath and are virtually absent in the brain before myelination and are present at abnormally low concentrations in any pathological conditions where severe loss of myelin occurs. It is practically absent in systemic organs except in the kidney, which normally contains appreciable amounts of galactosylceramide, although much less than in the nervous system.

Chemically, they consist of sphingosine, fatty acid, and galactose (Fig. 16)

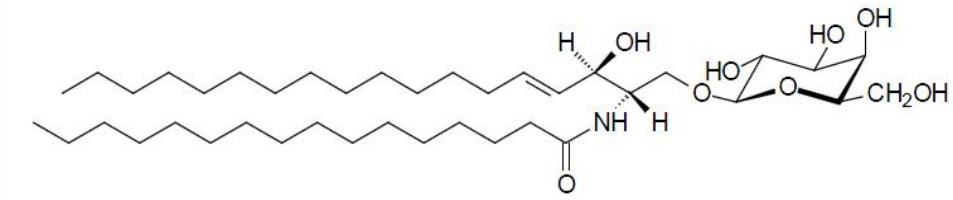

Fig. 16 Structure of galactosylceramide

The production occurs with a direct transfer of the carbohydrate moiety from a sugar nucleotide, e.g. uridine 5-diphosphate (UDP)-galactose, to the ceramide unit and it is catalysed by specific glycosyl-transferases, in this step the inversion of the glycosidic bond occurs (from *alpha* to *beta*). Synthesis of galactosylceramide takes place on the luminal surface of the endoplasmic reticulum, although it has free access to the cytosolic surface by an energy-independent flip-flop process (Christie, 2011).

The amount of galactosylceramide in immature brain is very low, because its biosynthesis reaches a peak during the most active period of myelination (during the first year and a half in humans and 15 days to 25 days in rodents), when myelin also turns over rather rapidly. In facts, activity of galactosylceramide synthase, UDP-galactose:ceramide galactosyltransferase (CGT) peaks sharply at 20–25 days after birth in rodent brains well correlating with the most active period of myelination (Costantino-Ceccarini and Morell, 1972).
During myelin turnover, galactosylceramide is broken down into galactose and ceramide by galactosylceramidase (Platt and Walkley, 2004) and these products are then used in a separate remyelination pathway (Suzuki, 2003).

In Krabbe disease, this breakdown does not occur and free galactosylceramide has a specific capacity to elicit infiltration of macrophages into the brain (Austin and Lehfeldt, 1965; Suzuki et al., 1976). Once in the brain, they phagocytize galactosylceramide and are transformed to multinucleated globoid cells. The characteristic inclusions in the globoid cells have morphological appearance identical to galactosylceramide itself (Yunis and Lee, 1970). No other agent is known to have a similar capacity *in vivo*. Therefore, galactosylceramide along with other myelin debris triggers a microglial response that results in the formation of globoid cells, a characteristic histopathological feature of Krabbe disease (Borda et al., 2008; Suzuki, 2003). In this way, the appearance of characteristic PAS-positive, electron dense and often multinucleated globoid cells, histopathological hallmark of Krabbe disease, are explained. However, loss of myelin-forming cells is not imputable to galactosylceramide, because if implanted in the brain does not exhibit any functionally detrimental capacity other than eliciting the globoid cell reaction: there is no experimental evidence that galactosylceramide is a metabolic toxin.

Meantime, galactosylceramide was never detected in patient's brain, explained by the early degeneration of the myelin forming cells and the block in the synthesis of galactosylceramide (Vanier et al., 1975; Svennerholm et al., 1980) and by the presence of GM1 gangliosidase (commonly known as β-galactosidase) capable of galactosylceramide hydrolysis (Kobayashi et al., 1985).

Psychosine

Psychosine is the trivial name for a monoglycosylsphingosine, which is the non-acylated or lyso form of a cerebroside, normally galactosylsphingosine. It is a minor intermediate in the catabolism of monoglycosylceramides, and is normally present in tissues at very low concentrations.

It is unusual in being a basic (cationic) lipid with a detergent-like structure, so it may have binding properties that differ from those of other lipids (Fig .17).

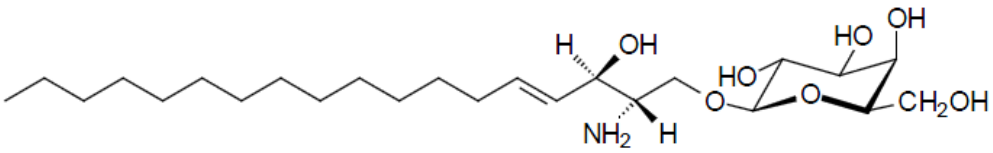

Fig. 17 Structure of psychosine

The biosynthesis is not very clear, because deacylation of the galactosylceramide would lead to formation of psychosine, although addition of galactose to sphingosine cannot be ruled out. Some evidences show, at least in mammalian tissues, psychosine can be generated only by galactosylation of sphingosine by galactosylceramide synthase, UDP-galactose:ceramide galactosyltransferase (CGT), but not by deacylation of galactosylceramide. Because CGT is nearly exclusively localized in the myelin-forming cells, synthesis of psychosine should also occur only in the oligodendrocytes and Schwann cells. It appears to be a dead-end product of cells during myelin turnover and it is broken down immediately by galactosylceramidase that is the only enzyme psychosine-hydrolyzing.

Consequently, psychosine is detectable in normal brain with highly sensitive analytical methods but its concentration is very small (less than 10 picomoles/mg protein), while in the Krabbe affected brain, psychosine is allowed to accumulate within the oligodendrocytes (Borda et al. 2008; LeVine, 1994; Suzuki, 2003) at very high levels. Based on this enzymological consideration, the “psychosine hypothesis”, first proposed by Miyatake and Suzuki (1972b), was then analytically demonstrated by several authors in the brain of patients (Vanier and Svennerholm, 1976; Svennerholm, Vanier, and Månsson, 1980) and in canine and murine models (Igisu and Suzuki, 1984). The “psychosine hypothesis” states that, not only the primary substrate of the defective enzyme, galactosylceramide, but also the toxic metabolite, galactosylsphingosine (psychosine), cannot be degraded and the consequent abnormal accumulation of psychosine causes the rapid loss of the myelin-forming cells (Fig. 18). The hypothesis initially met considerable skepticism but has survived the intervening 30 years (Suzuki, 1998), and the myelin debris, formed by toxic build-up of psychosine, triggers an inflammatory response of astrocytosis and

microgliosis (Suzuki, 2003). While galactosylceramide does not show toxic effects, psychosine is highly cytotoxic (Taketomi and Nishimura, 1964), both in animals where causes fatal hemorrhagic infarct when implanted into the brain (Miyatake and Suzuki, 1972b) and *in vitro* (Giri et al., 2006). The "psychosine hypothesis"' is also supported by a study conducted into the pattern of psychosine accumulation using Fourier-transform infrared microspectroscopy (FT-IR). FT-IR allows the accumulation of psychosine to be measured *in situ* (LeVine et al., 1994) during myelination stages.

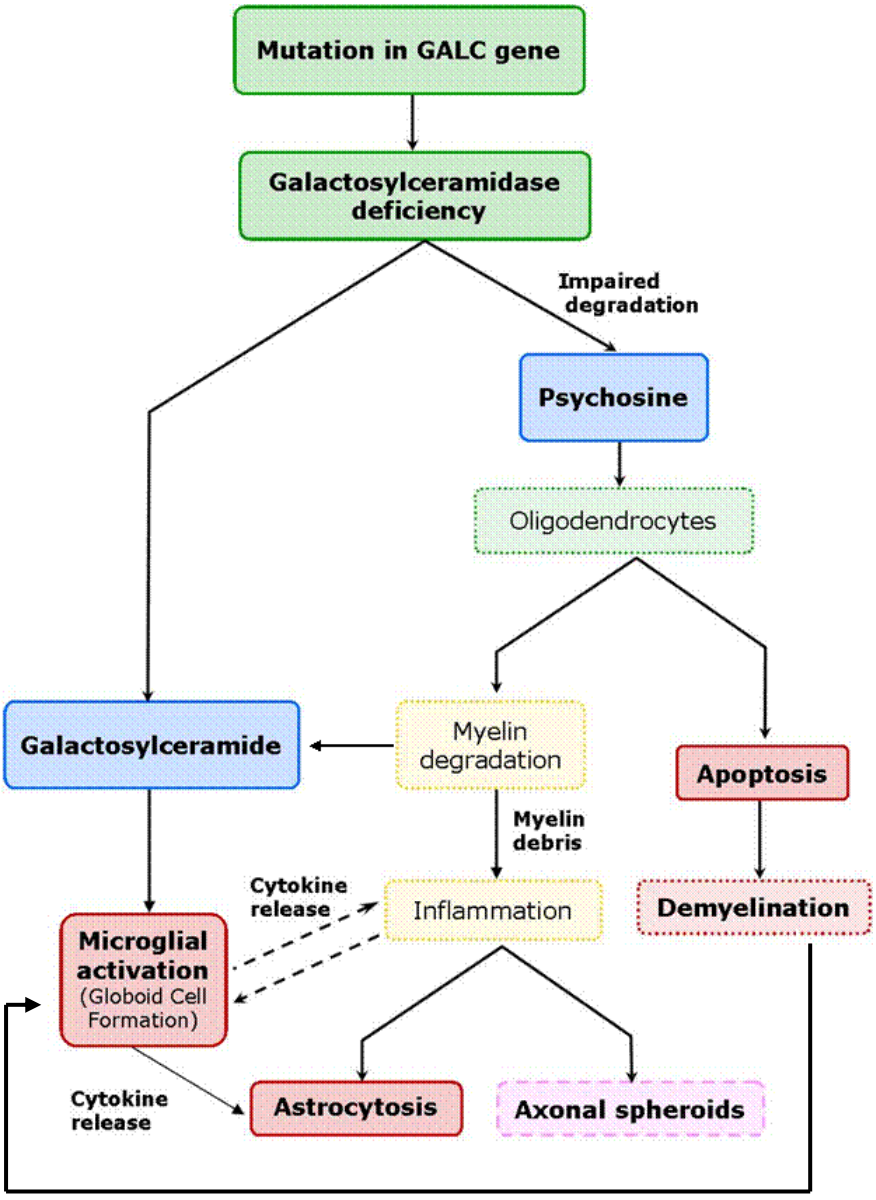

Fig. 18. Psychosine and galactosylceramide in pathogenic cascade of Krabbe disease
[From Fletcher et al, 2009 and adapted from Suzuki, 2003]

Following this hypothesis, research addresses in understanding the molecular mechanisms related to psychosine- inducted toxicity, even if they have not been yet fully elucidated.

Psychosine seems to interfere in different cellular systems. Toxic levels of psychosine have been shown to induce several signaling responses in cell culture that mimic Krabbe disease condition *in vivo* (Taniike et al., 1999; Jatana et al., 2002; Haq et al., 2003). These data were confirmed *in vivo* (Zaka and Wenger, 2004).

Cell death is shown associated to psychosine-induction of pro-inflammatory cytokines [tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6)] (Formichi et al., 2007), inducible nitric oxide synthase (iNOS) (Giri et al., 2002), phospholipase A2 (PLA2) activity and lysophosphatidylcholine generation (Giri et al., 2006). Moreover, down-regulation of phosphoinositide 3-kinase (PI3K)-Akt survival pathway (Zaka et al., 2005), expression of prostaglandin D synthase (Mohri et al., 2006a,b), and formation of multinuclear globoid like cells in U937 monocytic cells (Kanazawa et al., 2000) and natural killer cells (Maghazachi et al., 2004) was related.

Apoptotic cells and expression of apoptosis-related molecule such as TNF-α and its receptor 1 have been observed in *twitcher* mice and Krabbe disease patients' brains. This data confirms the hypothesis that induction of inflammation during progressive accumulation of psychosine is one of the main biochemical and pathogenetic mechanism of cell death in the Krabbe brain (LeVine and Brown, 1997; Taniike et al., 1999; Jatana et al., 2002). In addition, it down-regulates AMP-activated protein kinase (AMPK), inducing inflammation in astrocytes without directly affecting the cell death of oligodendrocytes (Giri et al., 2008); Hannun and Bell (1993) showed that psychosine inhibits the activity of protein kinase C (PKC).

The relationship between apoptosis and stress signals psychosine-mediated is also explained by MAPKs that act by down-regulating the prosurvival (NF-κB) and up-regulating the stress-mediated signal transduction pathways (JNK/AP-1) with or without the receptor function (Haq et al., 2003).

In fact, in 2001 the receptor for psychosine, the G protein-coupled orphan receptor TDAG8, was discovered (Im et al., 2001). Activation of the receptor in RH7777 hepatoma cells by psychosine and related lysoglycolipids results in an inhibition of forskolin-induced cAMP accumulation. It has also been suggested that, independent of TDAG8, a phospholipase A2 (PLA2)-mediated signal transduction pathway plays a critical role in the psychosine-induced cell death of oligodendrocytes (Giri et al., 2006).

Another hypothesis is that psychosine mediates several of its toxic effect via mitochondria, including inhibition of the electron transport chain, loss of mitochondrial membrane potential, alteration of oxidative phosphorylation, cytochrome c release and activation of caspase 3 and 9 (Tapasi et al., 1998; Haq et al.,2003).

The alteration of peroxisomal functions (Khan et al., 2005; Haq et al., 2006) due to psychosine action seems to be linked with TNF-α and IL-6 elevated expression (Contreras et al., 2008).

Many authors investigated on psychosine effects in hampering actin reorganization leading to the formation of microglia and macrophage-derived multinuclear globoid cells (Kanazawa et al., 2000; Kozutsumi et al., 2002; Ijichi et al., 2013). Kanazawa et al. showed that psychosine induces formation of multinuclear globular giant cells by cytokinesis inhibition and not by cell–cell fusion. Formation of giant clots of actin filaments near the plasma membrane was reported in psychosine–treated cells (2 μM). Psychosine has been identified as an endothelial actin disassembling agent endowed with anti-angiogenic activity *in vitro* and *in vivo*. Psychosine inhibits different steps of the angiogenesis process *in vitro*, including endothelial cell proliferation and migration (Belleri et al., 2013).

Different reports give to psychosine and apoptosis a key role in defecting axonal cytoskeleton and transport (Castelvetri et al., 2011; Smith et al., 2011; Cantuti-Castelvetri et al., 2012), and the same group demonstrates the psychosine-mediated alterations in membrane architecture (White et al., 2009, 2011). It was demonstrated psychosine accumulates in the lipid rafts in CNS and PNS of *twitcher* mice and Krabbe patients. This accumulation was accompanied by significant disruptions in the architecture and composition of lipid raft domains, leading to induction of giant clots of actin filaments and alterations in the distribution of the PKC signaling molecule (Fig. 19).

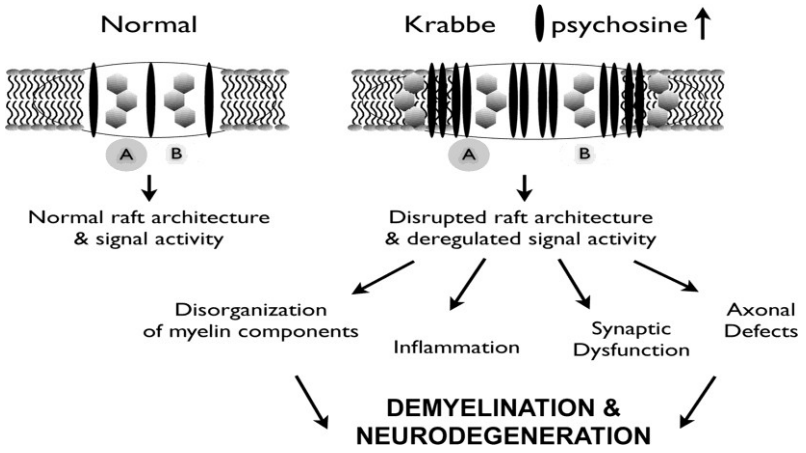

Fig. 19. Mode of psychosine accumulation in the raft domain
Under normal sphingolipid metabolism, two hypothetical partners (A, B) are spatially accommodated to provide functional interaction, hence, normal signal activity. In Krabbe disease, the raft domain increases in size because of the accumulation of psychosine and other components of the raft, leading to the spatial separation of the two partner molecules A and B, disrupting the function of the associated signal. [From White et al., 2009]

Chemical lipid raft disruption can result in loss of function of associated signaling molecules, and it is possible that the mechanistic link between psychosine inhibition of PKC and the decreased PKC activity in Krabbe's disease is the psychosine-based disruption of the membrane architecture. White et al. (2009) reported that psychosine accumulation in lipid rafts has functional consequences that range from interference with cell signaling to deregulation of raft-mediated endocytosis. For these reasons, traditional GALC replacement therapies in *twitcher* mice are insufficient to remove psychosine from these domains and eliminate disease. Thus, psychosine toxicity is mediated by membrane perturbation (Hawkins-Salsbury et al., 2013).

However, further research is required before a solid conclusion can be drawn on these pathogenic mechanisms.

EXPERIMENTAL DESIGN

"Nullis in verba"[2]

London Royal Society (1660)

[2] The latin motto of Royal Society of London states a rejection of the principle of authority and a determination to establish facts through observation and experimentation, and thus to practise objective science, independently from political or religious influences.

SPECIFIC AIMS

Krabbe disease is an autosomal recessive disorder resulting from the genetic deficiency of galactosilcerebrosidase (GALC), a lysosomal enzyme involved in the catabolism of two lipids extremely represented during formation of myelin sheet: galactosylceramide and psychosine. For this reason the pathology is classified as a lysosomal storage disease with the peculiarity the primary GALC substrate, galactosylceramide, does not accumulates in the brain of Krabbe patients, meantime psychosine do it at very high levels and causes a strongly demyelization due to loss of myelin-producing cells. Since 1972, when these conditions were postulated in the "psychosine hypothesis", researchers are trying to define mechanisms occurring as consequences of metabolite accumulation in white matter. In order to achieving the goal not only cell culture, but also animal models were used. Especially, the mouse naturally occurring model of globoid cell leukodystrophy, known as twitcher mouse for its rapid twitching at symptoms appearance, is well characterized and has potential for studies about evaluating pathogenetic mechanisms and testing novel treatment options for Krabbe's disease, which could not be ethically obtained in a human. Still today, therapies investigated in the model mice limited their success only in delayer onset of the symptoms, while axonopathy and caspase-3 activation are reported as psychosine- mediated actions.

In human, the clinical phenotype is extremely severe for the extensive cellular dysfunction, brain deterioration and irreversible neurological handicap with motor- and mental-degeneration. In addition, ninety per cent of all cases present the infantile form and the exit is dramatic for the early death at an average of two years old. Currently, the only clinical treatment used is bone marrow transplant (BMT), which must be performed before symptoms appear to be effective and this procedure is also a risky and is extremely uncomfortable for anyone, especially for newborn children. The morbidity and mortality associated with BMT, along with the fact that there is no law obligating the KD screening at bird and in most of cases the diagnosis occurs after symptoms appear, they suggest that KD have no a cure.

Moreover, in this period the stem-cell therapy applied to neurological disease is more and more under discussion, highlighted by current events in Italy. A Brescia-based organization called the Stamina Foundation used and is promoting an unproven mesenchymal stem cell therapy to vulnerable patients, including children with lethal neurological diseases, and in order to escape the European regulations on stem-cell therapies, MSC infusion were reported as compassionate therapy. Avoiding to analyse in this context the governmental actions, the economical interests and the personal history of Stamina's president (see *Nature* **499,** 125; 2013), this Italian case

take worldwide attention on the necessity to discriminate between advertisement and science and commerce (see Bianco In Nature 499, 225; 2013).

This study was developed during three years of Ph.D. school in Neuroscience at University of Catania and three main questions were investigated by the creation of *in vitro* models and investigating pathways comparable to situation occurring in *twitcher* mice and Krabbe disease patients. The questions were the followings:

1. Compared to others cells, is there a specific psychosine-mediated pathway that is activated in oligodendrocytes precursors cells?
2. Have oligodendrocytes progenitor cells, obtained from wild type and *twitcher* mice brain, the same capacity to differentiate in mature cells?
3. Can mesenchymal stem cells from adipose tissue be differentiated towards a neural/glial-like phenotype in order be used for cell replacement therapy?

Psychosine is unusual in being a basic (cationic) lipid with a detergent-like structure. Thus, it may have binding properties that differ from those of other lipids and potential for acting in different ways, e.g. through modifications in the fine membrane structure, cell–cell or cell–matrix interactions, and/or modulation of intracellular signaling in order to promote apoptotic loss of myelin-forming cells.

First, we hypothesized that differences in cell responses to addition of exogenous psychosine could be related to cellular type. Therefore, different assays were carried out for detecting the psychosine-mediated effects on viability of wild type and GALC enzyme-modified oligodendrocytes progenitors and fibroblasts.

In order to investigate the psychosine-activated molecular signalling, the phosphatidylinositide 3-kinases (PI3K)/Akt pathway was studied. This pathway is involved in essential cellular processes, such as proliferation, differentiation, migration, but also it orchestrates the programmed-cell death or apoptosis.

Other biological molecules offering an interesting field of research were the connexins. Usually, the connexin family members are known as constituent of Gap junctions (GJs). Recently, a key role in apoptosis and in regulation of cellular conditions with new evidence in GJ-independent function is reported for Connexin43. Still today, no study was performed for evaluating Cx43 expression in Krabbe disease. We hypothesized a possible involvement of Cx43 in cell death induced by psychosine and maturation of oligodendrocytes progenitors. Therefore, its expression was investigated in oligodendrocytes progenitors with and without psychosine or in presence of differentiating medium.

Finally, it was probed the likelihood of using adipose tissue-derived mesenchymal stem cells (AT-MSCs) as source of oligodendrocytes. Cell response to conditioned-media obtained from different cell lines was detected for defining AT-MSCs sensitivity to environmental factors.

The major objective of this study was a more deep comprehension of the underlying biochemical mechanism induced by psychosine in cellular systems and in *twitcher* mouse model. The isolation and characterization of precursor oligodendrocytes cells and fibroblasts was made from wild type or GALC-mutated mouse brains and tails, respectively. Developing of a stable oligodendrocyte precursor cell line from *twitcher* and wild type mice that could undergo differentiation *in vitro* was the first principal aim. Such cell culture system could be a useful tool to study the pathological mechanisms that induce oligodendrocytes degeneration and verify novel therapeutic approaches.
After validation of cell systems, the aims of this study were the followings:

1. The comparison of the effects and the pathways related to psychosine-treatment in the all cellular models used.

2. The determination of different pathways activated by the psychosine wich might lead to apoptosis in defined experimental condition.

3. The detection of Cx43 expression in untreated and psychosine-stimulated cells.

4. The evaluation of intracellular Cx43 distribution of psychosine-treated oligodendrocytes progenitors compared to untreated controls.

5. The assessment of Cx43 levels during differentiation steps of wild type or *twitcher* mouse oligodendrocytes precursor cells.

Finally, in isolated and well characterized adipose tissue-derived mesenchymal stem cells (AT-MSCs), it was examined the potential effects of B104- or OECs-conditioned media in differentiation of AT-MSCs towards a glial-like phenotype.

MATERIALS AND METHODS

Animals

Animal handling protocols used in this study has been approved by the Institutional Animal Care in accordance with institutional guidelines for animal care and use. Pairs of twitcher mice, a strain that contains a premature stop codon (W339X) in the galactosylceramidase (GALC) gene that abolishes enzymatic activity, was acquired from Arlan laboratories, as gift from "Progetto Grazia", an ONLUS Italian Association for Research on Krabbe disease.association.A colony was subsequently bred and maintained in our facility. The animals were housed in plastic cages in a pathogen-free room with controlled temperature (22 ± 2 °C), relative humidity (45 ± 5%) and 12/12 h light/dark cycle. Food and water were available ad libitum and the cages cleaned once a week. The genotypes of pups born from the twitcher heterozygotes were determined by polymerase chain reaction (PCR) on genomic DNA extracted from clipped tails according to the method reported by the manufacture of kit (Sigma Aldrich, Milan, Italy).

Oligodendrocytes precursor cells (OPCs) isolation

The procedure for the isolation of oligodendrocyte progenitor cells has been described in Strazza et al., 2004. Briefly, the primary cultures of wild-type and twitcher OPCs were prepared from pooled cerebra 2-days old C57BL6J mice. Cerebral cortices were dissected out, minced, digested for 15 min at 37 ◦C in MEM (Gibco, Grand Island, NY) containing 0.1% trypsin (Sigma), and triturated after adding 60μg/ml DNase I (Sigma) and 5% fetal bovine serum (FBS, Lonza). Dissociated cells were sequentially passed through nylon meshes of 210-, 130-, and 35-mm pore sizes (35) and plated at the concentration of one brain per T-25 flask coated with 100μg/ml polyl-lysine (Sigma). Cell cultures were maintained in DMEM containing 10% fetal bovine serum at 37 ◦C in humidified air with 5% CO_2 for 10 days, with a medium change every 3 days. After, the cells were rinsed three times with culture medium, were sealed and shaken overnight at 200 rpm on a rotary shaker at 37 ◦C. On the next morning, the medium with the detached cells was collected and first plated on tissue culture dishes (Corning, NY) for 30 min at 37°C with a gentle swirling of the dishes after 15 min to eliminate contaminating astrocytes and microglia. The non-adherent cells were collected and replated in DMEM containing 10% FBS at densities between 50,000 and 2000 cells/cm^2 onto 96 well tissue culture dishes or coverslips poly-l-lysine coated, and the following morning, the growing medium was added. Oligodendrocytes were purified from contaminating astrocytes by immunopanning on plates coated with A2B5 antibodies. Oligodendrocyte precursors were obtained after removal of residual astrocytes. The clonal cell lines were obtained from this primary oligodendrocytes cultured for

extended time in a defined medium containing PDGF-A and basic FGF, in fact mouse cells undergo spontaneous transformation with time. The OPC-WT and OPC-TWI were cloned by limited dilution and further purified by positive immunoselection. Immunopanning on the A2B5 antibody was made because this glycolipid is present on the membrane of oligodendrocyte progenitors. Cell culture basal media, trypsin and all other supplements were purchased from Lonza (Long Island, NY), PeproTech Inc. (UK) or Sigma (St. Louis, CA).

OPCs culture condition

$A2B5^+$ cells underwent maximum cell death in the absence of B104-conditioned medium and other mitogens and could not be induced to differentiate into O4-positive OL (cell marker for mature OL) *in vitro* using several different growth conditions. OPCs were cultured in polylysine-coated plates with DMEM medium supplemented with 0.29 g/l L-glutamine, 1.5% non-essential amino acids, 6 g/l glucose supplement, 50 μg/ml gentamicin, 30% B104-conditioned medium, 2% B-27 supplement, 5 μg/ml insulin, 15 nM thyroid hormone (T3), 10 ng/ml basic fibroblast growth factor (bfGF) and 10 ng/ml platet-derived growth factor A (PDGF-A). The media was replaced on alternative days.

OPCs differentiating medium

Various differentiation media were assayed, but the best results were obtained with a simple correction of growing medium: PDGF-A and bFGF were withdrawn from the described culture medium, as previously described (Yang et al., 2005), and 0.537mg/l of L-Thyroxin was added.

Fibroblasts

Fibroblasts were obtained from tails of normal and *twitcher* C57BL6J mice. Briefly, tails were clipped in small pieces; always avoid drying by refreshing tissue with PBS. Five – six pieces were added in a T-25 cm^2 flask and maintained without medium at 37°C in humidified air with 5% CO_2 overnight. Next morning DMEM (Lonza) containing fetal calf serum (FCS, 10%), L-glutamine (2.0 mM), penicillin (100 UmL^{-1}), streptomycin (100 μgmL^{-1}) was added and changed every 3 days. After approximately 15 days, the cells appeared and the tissue was removed.

Psychosine treatment

Psychosine from bovine brain (P9256, lyophilized powder, P98%, Sigma, Milan, Italy) was dissolved in ethanol at the concentration of 10 mM and was further diluted in treatment media to the indicated concentrations. For experiments, 24 h before the OPCs were trypsinized, counted in a haemocytometer, and plated in polylysine-coated 96 (for MTT test) wells plates or in 100 mm Petri-dishes (for Western blot). In this case, cells were incubated in treatment media (complete media excluding insulin, PDGF-A, bFGF and T3 or serum) for 24 h prior to the various treatments with psychosine.

Cell viability determination

Cell viability was evaluated by determining mitochondrial function of living cells on the basis of their ability to reduce the yellow dye, tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma), into dark blue formazan crystal mainly by the mitochondrial dehydrogenases (Mosmann T., 1983). Cells were seeded at the density of 8.5×10^3 cells/well in 96-well plates and incubated at 37 °C in an atmosphere of 5% CO2 for 24h with growing medium. Then cells were pre-incubated with the treatment medium for an additional 24h. Cells were then treated with different concentrations of psychosine for 24 h. 200 ml of MTT in PBS at final concentration of 0.5mg/ml was added into each well for 3 h at 37°C. The dimethylsulphoxide was addede into each well as solubilisation solution for the blue formazan product. The absorbance of solubilized formazan solution was measured by a microplate spectrophotometer reader (Titertek Multiskan, DAS, Italy) at 550 nm. For each sample three experiments in triplicate were performed and percentage of cell viability of treated cells was compared with that of untreated control cells.

Cell growth analysis

Cells were plated at 2×10^4 cells/well density. Cells from each well of the triplicate were trypsinized and counted daily, and the mean number of viable cells /well was obtained every day from the triplicate average. Trypan blue test was always performed.

Apoptosis assay

Cells approaching 70% confluence were serum or growth factor starved for 24 h and then incubated with 50 μm psychosine. Twenty hours later, the cell culture medium (containing dead cells) was aspirated and combined with trypsinized (live) cells, which were then pelleted at 1400 x g and fixed in 3% paraformaldehyde for 2 h at 4 C.Cells were cytospun onto poly-l-lysine coated slides (5 min, 1400 x g). Nuclear DNA was stained with 4,6-diamidino-2-phenylindole (DAPI, 1:10 000; Invitrogen). Digital images were acquired using a Leica DMRB fluorescence microscope (Leica Microsystems Srl, Milan, Italy) equipped with a computer-assisted Nikon digital camera (Nital SpA, Turin, Italy) with filter at 350nm. More than 1000 single cells per replicate were scored using chromatin fragmentation as an indication of apoptosis. Each condition was assessed in triplicate; means of the triplicate counts were calculated, and values expressed as Apoptotic Index (AI): number of apoptotic cells in 1000 nuclei counted (mean ± sem).

E.L.I.S.A.

A sandwich ELISA was used for detecting active caspase-3, following producer's instruction. Briefly, cell lysates were added in top of microwells and the plate incubated for 2 h at 37°C. After gently removing the tape, wells were washed 4 times with 1X Wash Buffer, and 100 μl of reconstituted detection antibody were added to

each well, followed by incubation at 37°C for 1 h. After, the wash procedure was repeated and 100 µl of reconstituted HRP-Linked secondary antibody was added to each well and the plate incubated for 30 min at 37°C.Additional washes were performed, following by 10 min at 37 °C incubation with 100 µl of TMB Substrate. After, 100 µl of stop solution were put in each well and the absorbance was read at 450 nm within 30 min after adding stop solution. Value was reported as average colour intensity of three independent experiments performed in triplicate.

Western blot

Proteins were extracted with lysing buffer (10 mM Tris–HCl plus 10 mM KCl, 2 mM MgCl2, 0.6 mM PMSF, and 1 % SDS, pH 7.4) enriched with Protease and Phosphatase Inhibitor Cocktail Tablets (Roche Applied Science) and were quantified using Bicinchoninic acid assay (Pierce,USA). Equal amount of protein (40 µg) was loaded to each well of Bolt™ 4-12% Bis-Tris Plus Gels (Invitrogen). After electrophoresis , proteins were transferred to a nitrocellulosa membrane, in a wet system, and proteins transfer was verified by staining membranes with Ponceau S. Membranes were blocked with Tris buffered saline containing 0.01% Tween-20 (TBST) and 5% non-fat dry milk at 4 °C for 1 h, and then incubated with primary antibody at 4 °C overnight. After washing three times with TBST (Tris-buffered saline with 0.1% Tween-20) buffer, membranes were incubated with the secondary antibody horseradish peroxidise (HRP)-conjugated (Santa Cruz), at room temperature for 1 h. The signals were developed by enhanced chemiluminescence detection Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL) and visualized with autoradiography film. Bands were measured densitometrically and their relative density calculated based on the density of the β-actin or α–tubulin (Sigma) band in each sample. Values were expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity. Primary antibodies used were anti-Connexin43 (1:5000 diluition ; Sigma–Aldrich), -p53 (1:300 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), -PI3K (1:200 dilution, Santa Cruz Biotechnology), -PTEN (1:1000 dilution, Sigma–Aldrich); -Bad (1:2000 dilution, Sigma–Aldrich), -Active Caspase3 (1:2000 dilution, Sigma–Aldrich), -NF-kB p65 (1:1000 dilution, Cell Signalling).

Immunocytochemistry

For cell staining, OPCs were cultured in a Poly-L-Lysine-coated coverslip for 24 h. The medium was removed and replaced with treatment medium for 24 h. Then, psychosine or the differentiating medium were added for 24h or 72h, respectively. The cells were first washed with PBS, then fixed with paraformaldehyde (4%) in PBS for 20 min. After washing with PBS, cells were incubated with a blocking solution containing 5% normal goat serum (NGS) for 1 h at RT, then permealized with Triton for 5 minutes. Primary antibody were incubated overnight in a humid room. The following day, cells were washed with PBS and incubated for one hour at room temperature with secondary antibody conjugated to different fluorochromes

and , after, with diamino-2-phenylindole (DAPI; 1:10 000; Invitrogen) for 10 min. A Mounting rapid solution was used for cover glass preparation. As a control, the specificity of immunostaining was verified by omitting the primary or secondary antibody. Digital images were acquired using a Leica DMRB fluorescence microscope (Leica Microsystems Srl, Milan, Italy) equipped with a computer-assisted Nikon digital camera (Nital SpA, Turin, Italy). Primary antibodies used were anti-A2B5 (1:5; Abcam, Prodotti Gianni, Milan, Italy), -Connexin43 (1:300; Sigma, Milan, Italy), -O1 and –O4 (1:5, medium from hybridom cells). They were Cy3-conjugated goat anti-rabbit or FITC- conjugated goat anti-mouse secondary antibodies (Millipore, Milan, Italy).

Patients

Adipose tissue was gathered from ten donors, five men and five women (from 22 to 30 years of age and mean body mass index of 27 + 3.8) undergoing abdominal liposuction procedures. Lipoaspirates were obtained under an approved Institutional Review Board protocol and after informed consent had been obtained from the patients at the Cannizzaro Hospital, Catania (Italy). The patients were not smokers and occasionally taking non-steroidal antiinflammatory drugs (NSAIDs). The women did not take estrogens replacement therapy.

Cultures of human MSCs from adipose tissue

As previously described (Lo Furno et al., 2013) the raw lipoaspirate (50–100 ml) was washed with sterile phosphate-buffered saline (PBS; Invitrogen, Milan, Italy) to remove red blood cells and debris, and incubated for three hours at 37 °C with an equal volume of serum-free Dulbecco's modified Eagle's medium (DMEM)-low glucose (DMEM-lg; PAA Laboratories, Pasching, Austria) containing 0.075% of type I collagenase (Invitrogen, Milan, Italy). Collagenase activity was then inactivated by an equal volume of DMEM-1g containing 10% of heat-inactivated fetal bovine serum (FBS; Invitrogen, Milan, Italy). Successively, the digested lipoaspirate was centrifuged at 1200 rpm for 10 min. The pellets were re-suspended in PBS (plus penicillin/streptomycin 1%) and filtered through a 100-mm nylon cell strainer (Falcon BD Biosciences, Milan, Italy). The filtered cells were again centrifuged at 1200 rpm for 10 min, plated in T-75 culture flasks (Falcon BD Biosciences, Milan, Italy) with DMEM-1g (10% FBS, penicillin/streptomycin 1%) containing 1% of MSC growth medium (MSCGS; ScienCell Research Laboratories, Milan, Italy) and incubated at 37 °C with 5% CO_2. Twenty-four hours after the initial plating, non-adherent cells were removed by intensely washing the plates.

Determination of MSCs markers

In order to identify MSCs derived from lipoaspirate, immunocytochemical procedures were carried out using several cell surface markers. Particular care was taken to distinguish MSCs type from hematopoietic stem cells.

After reaching confluence (80% of total flask surface), all subpopulations were trypsinized (Sigma-Aldrich, Milan, Italy) and subcultured in 12-well culture dishes for two days. For immunocytochemistry, cells were first washed with PBS, then fixed with 4% paraformaldehyde in PBS for 30 min and incubated for 30 min with a 5% solution of normal goat serum (NGS; Sigma-Aldrich, Milan Italy). They were subsequently incubated overnight at 4 °C with primary antibodies (Millipore, Milan, Italy): CD44, 1:200 dilution; CD90, 1:100; CD105, 1:100; CD14, 1:200; CD34, 1:200; CD45, 1:200. The following day, cells were washed with PBS and incubated for one hour at room temperature with secondary antibody conjugated to different fluorochromes. They were fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or Cyanine (Cy)3-conjugated goat anti-mouse secondary antibodies (Millipore, Milan, Italy). As a control, the specificity of immunostaining was verified by omitting the primary or secondary antibody. As a rule, cell nuclei were counterstained with DAPI for 10 min.
Digital images were acquired using a Leica DMRB fluorescence microscope (Leica Microsystems Srl, Milan, Italy) equipped with a computer-assisted Nikon digital camera (Nital SpA, Turin, Italy). The excitation wavelength was 554 nm for Cy3, 488 nm for FITC and 350 nm for DAPI. Immunostaining was evaluated taking into account the signal-to-noise ratio of immunofluorescence.

OECs cultures and preparation of OECs-CM

As previously described (Pellitteri et al., 2009), Olfactory Ensheathing Cells (OECs) were isolated from 2-day old rat pups (P2) olfactory bulbs. Briefly, pups were decapitated and the bulbs were removed and dissected out in cold (4 °C) Leibowitz L-15 medium (Sigma–Aldrich, Milan, Italy). Subsequently, they were digested in Medium Essential Medium-H (MEM-H, Sigma–Aldrich, Milan, Italy) containing collagenase and trypsin. Trypsinization was stopped by adding DMEM supplemented with 10% fetal bovine serum (FBS, Sigma–Aldrich, Milan, Italy). Cells were resuspended, planted in flasks and fed with fresh complete medium DMEM supplemented with 10% FBS. The antimitotic agent, cytosine arabinoside (10^{-5} M), was added 24 hours after initial plating to reduce the number of dividing fibroblasts. A method to further purify the samples was adopted following the method by Chuah and Teague (1999): OECs cultures were processed to an additional step, transferring cells from one flask to a new one. This step reduces contaminating cells, because they adhere more readily to plastic than OECs. In the last passage, OECs were plated on 25 cm^2 flasks and cultured in DMEM/FBS supplemented with bovine pituitary extract. Cells were incubated at 37 °C in fresh complete medium and were fed twice a week. OECs were characterized by immunocytochemistry using S-100 as a marker (Pellitteri et al., 2009).
OECs were cultured to confluence and the medium was collected after approximately 24–48 hours. OECs-CM was filtrated (0,45 μm filter) to remove detached cells, aliquoted and stored at -20 °C until further use.

B104 cultures and preparation of B104-CM

The B104 neuroblastoma cell line was maintained in logarithmic phase of growth in DMEM supplemented with 10% heat-inactivated FBS, 2 mM of glutamine and 100 μg/ml penicillin/streptomycin.

For B104-CM preparation, B104 cultures (density: 100-150 cells/mm^2) were washed twice in PBS and incubated in serum-free DMEM containing 1x N1 supplement (N6530; Sigma–Aldrich, Milan, Italy), 2 mM of glutamine and 100 μg/ml penicillin/streptomycin. After three days, the medium was collected, filtered (0,45 μm), aliquoted and stored at -80 °C.

MSCs treatments

After reaching confluence (80% of total flask surface), all subpopulations were trypsinized (Trypsin-EDTA; Sigma-Aldrich, Milan, Italy) and subcultured in 6 multi-wells plates for twenty-four hours. The medium was removed and replaced with OECs-CM and/or B104-CM. Some wells were used as control and incubated with DMEM supplemented with 10% FBS. Cells were then incubated for one and seven days. Some samples were used for immunocytochemistry and others for flow cytometry.

Effects of OECs-CM or B104-CM treatment

To order to verify if OECs-CM or B104-CM was capable of inducing differentiation of AT-MSCs to a neuronal or glial phenotype, some specific markers, such as nestin, protein gene product 9.5 (PGP 9.5), microtubule-associated protein 2 (MAP2), glial fibrillary acidic protein (GFAP) and neuron cell surface antigen (A2B5) were examined by immunostaining and flow cytometry 24 hours and 7 days after the treatment.Cells were trypsinized, fixed with 2% paraformaldehyde (PFA) for 20 minutes at 4 °C and permeabilized with 1x Triton (Sigma-Aldrich, Milan, Italy) for 5 minutes at 4 °C. Afterward, cells were washed once with PBS/BSA 1% and incubated with anti--nestin (1:600; Abcam), -PGP 9.5 (1:600; AbD Serotech), MAP2 (1:200; Covance), -GFAP (1:200; Abcam), and -A2B5 (1:5; Abcam) antibodies for 60 minutes at room temperature. Cells were then washed once with PBS/BSA 1% and incubated with goat anti-mouse or goat anti-rabbit conjugated with FITC (1:200; Millipore, Milan, Italy) for 60 minutes at room temperature in the dark. Samples were analyzed using a Coulter Epics Elite ESP flow cytometer (Coulter, Miami, FL, USA). A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at λ= 488 nm and fluorescence was monitored at λ= 525 nm. Fluorescence was detected using logarithmic amplification. Mean fluorescence intensity (MFI) values were calculated and recorded. For cell staining, the experimental cells were cultured in a 12-well culture dishes for 24 h. The medium was removed and replaced with medium without phenol red containing the dispersion of 3 with for 12 h. The cells were first washed with PBS, then fixed with paraformaldehyde (4%) in PBS for 20 min. After washing with PBS, cells were incubated with a blocking solution containing 5% normal goat serum (NGS) for 1 h

at RT, then permealized with Triton for 5 minutes. Primary antibodies were incubated overnith in a humid room. The following day, cells were washed with PBS and incubated for one hour at room temperature with secondary antibody conjugated to different fluorochromes and, after, with diamino-2-phenylindole (DAPI; 1:10 000; Invitrogen) for 10 min. A Mounting rapid solution was used for cover glass preparation. As a control, the specificity of immunostaining was verified by omitting the primary or secondary antibody. Digital images were acquired using a Leica DMRB fluorescence microscope (Leica Microsystems Srl, Milan, Italy) equipped with a computer-assisted Nikon digital camera (Nital SpA, Turin, Italy). Primary antibodies used were anti-nestin (1:600; Abcam, Prodotti Gianni, Milan, Italy), -PGP 9.5 (1:600; AbD Serotec, Space Import-Export srl, Milan, Italy), -MAP2 (1:200; Covance, c/o Regus Business Centre, Rome, Italy), -GFAP (1:200; Abcam, Prodotti Gianni, Milan, Italy), and -A2B5 (1:5, Abcam, Prodotti Gianni, Milan, Italy) overnight.. They were FITC-conjugated goat anti-rabbit or Cy3-conjugated goat anti-mouse secondary antibodies (Millipore, Milan, Italy).

STATISTICAL ANALYSIS

The results were expressed as mean ± SD based on data derived from three independent experiments run in triplicate. Statistical analysis of results was performed using Student's t test and one-way ANOVA test plus Dunnett's test by the statistical software package Graphpad. P value lower than 0.05 was considered statistically significant.

RESULTS

A great amount of reports about Krabbe disease was made by analysis of tissue from *twitcher* mouse or Krabbe patients. In this way, the psychosine effects were described in a system very complex where some little details could be never found. Different signals from many cellular pathways, in fact, could cooperate in the extreme tentative to restore psychosine-mediated damages. Instead, cellular models are often more useful for detecting peculiar difference in cell response systems, especially in all cases when pathogenesis is not fully elucidate, as in Krabbe disease.

Based on these considerations, oligodendrocytes clonal cells were obtained from *twitcher* and wild type mice. Such cell cultures are not easily available, but double useful for both the investigation of the Krabbe disease pathogenesis and screening of new therapies.

Primary oligodendrocyte cultures from 2-days old wild type and *twitcher* mice were prepared. Purified primary cultures of oligodendrocyte precursors were obtained by positive immunoselection with the specific antibody to A2B5. After repeated passages in presence of growth factors, two stable progenitor cell lines were established: the OPC-WT cell line obtained from wild type mice and the OPC-TWI from the *twitcher* mice. These cells were expanded *in vitro* and their properties remained stable upon passaging. These cells are birefringent under the optical microscope and show the spindle shape morphology typical of the oligodendrocyte precursors (Fig. 20).

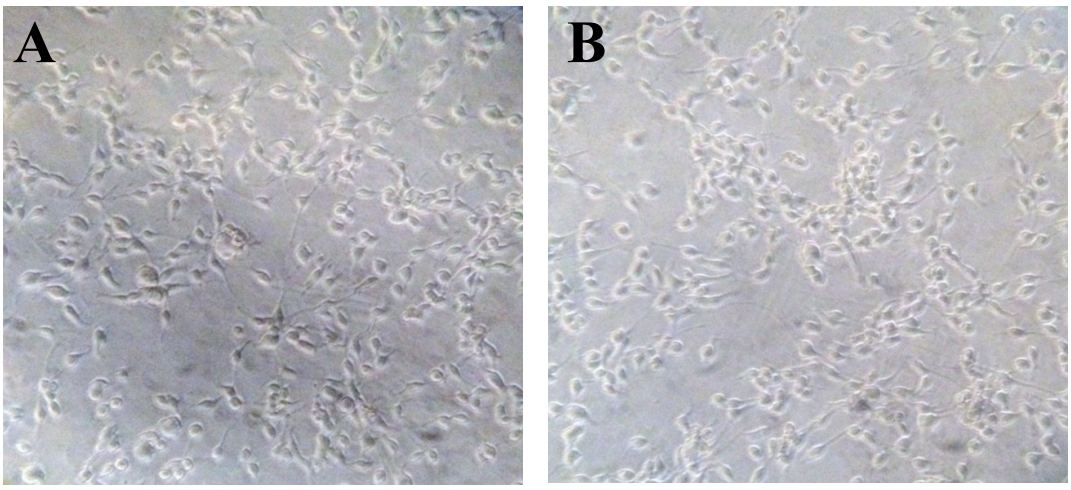

Fig. 20. Oligodendrocyte progenitors obtained from wild type (A) and *twitcher* (B) mice brain
Immunoselection with the specific antibody to A2B5 was made.

To evaluate the effect of psychosine on cell viability, OPC-WT and OPC-TWI cells were exposed to different psychosine concentrations for 24 hours in a medium

without insulin and growth factors to avoid any interference in cell survival. Cell growth was quantified using the MTT assay.

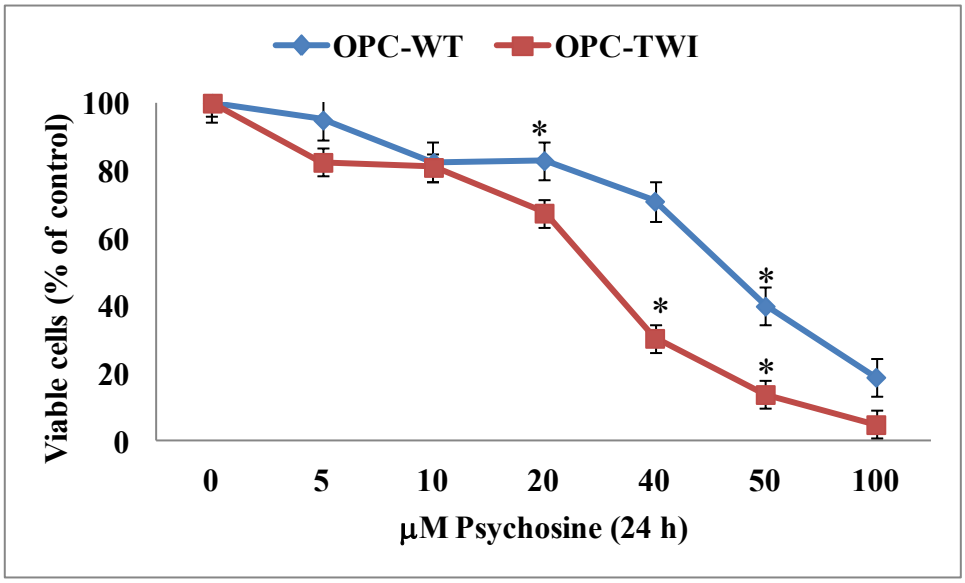

Fig. 21. Psychosine treatment in oligodendrocyte progenitors
OPC-WT- and -TWI were treated with different concentrations of psychosine.After 24 h, MTT assay was performed.
Each point represents mean ± S.D. of three separate experiments performed in triplicate.

The results on OPCs showed that the exposure to increasing concentrations of psychosine resulted in a decrease in cell viability in both cell types with some significant differences: 50 µM of psychosine induced up to 60% and 90% cell death in OPC-WT and OPC-TWI, respectively (Fig. 21). The highest dose (100 µM) determined a full loss of mitochondrial dehydrogenases activity in GALC deficient OPC-TWI after 24 hours of treatment. Under the same experimental conditions, OPC-WT cells maintained a partial mitochondrial enzyme activity that was never higher than 20% versus untreated control.

These data suggest that the psychosine-inducted toxicity in oligodendrocyte progenitors was dose-dependent on both progenitors, but in the OPC-TWI cells, the effects of psychosine were stronger and reached in a shorter time, resulting in significantly higher loss of cell viability.

Moreover, in this study, FIBRO-WT viability was compared to FIBRO-TWI, after 24 h treating with increasing concentrations of psychosine.

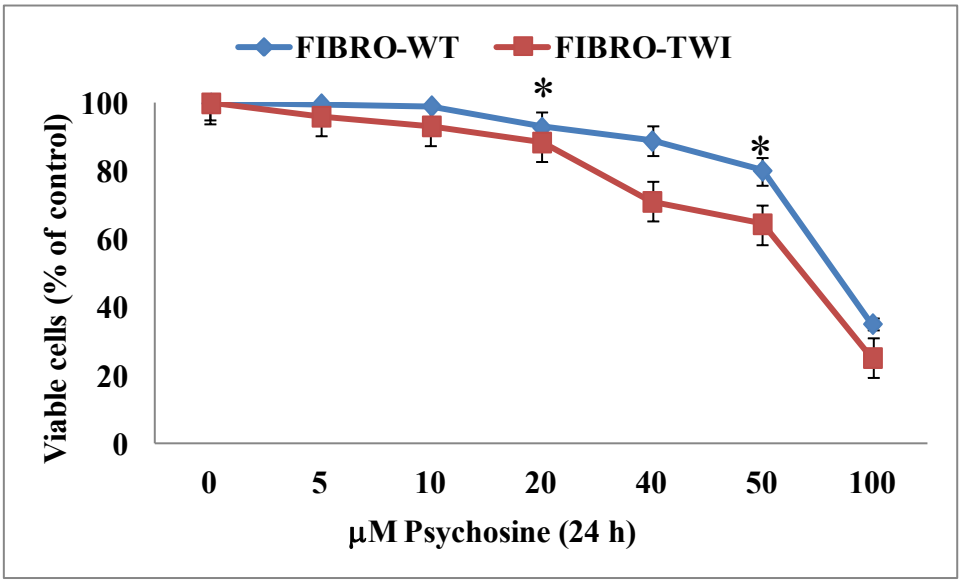

Fig. 22. Psychosine treatment in wild type and GALC-mutated fibroblasts
Cells were treated for 24 h with different concentrations of psychosine.
Cell viability was assessed using the MTT assay.
Each point represents mean ± S.D. of three separate experiments performed in triplicate.

As shown in Fig. 22 both fibroblasts respond to psychosine in a dose-dependent manner, but the values of cellular viability never reached those of OPCs, in all conditions examined. Compared to untreated controls, 50 µM of psychosine determined a viability reduction up to 20% and 40% in FIBRO-WT and FIBRO-TWI, respectively. The highest concentration resulted in absorbance values showing no significant difference in both type of fibroblasts.

The data show that there is a different sensitivity to the action of psychosine in cell models used, but, no matter cell type used, high concentration of psychosine is responsible for dramatic reduction in cell viability.The most interesting data derived from FIBRO-TWI treatment: even if cells presented the same GALC deficiency than OPC-TWI, a high resistance to psychosine action was detected.

These results fit well with the "psychosine hypothesis". In both OPC cell models psychosine is fully effective in killing cells at a concentration of 40 µM corresponding to 40 nmoles/ml. Considering that psychosine concentration in normal brains is 2-3 nmoles/gram and it is increased up to 10-20 fold in Krabbe patient and in *twitcher* brains, the scheme of dose-response showed in our models could perfectly reflect the condition of brain oligodendrocytes, when psychosine is preferentially accumulated.

Thus, the next steps were addressed to investigation of molecular mechanisms capable of explaining the significant difference in cell sensitivity to psychosine exposure.

The MTT assay measures mitochondrial integrity, but it does not indicate whether cells are arresting their growth or they are dying for apoptosis. Thus, after 24 hours of psychosine treatment, the normal maintaining condition were restored for 48 hours and a cell growth curve was obtained by counting viable cells.

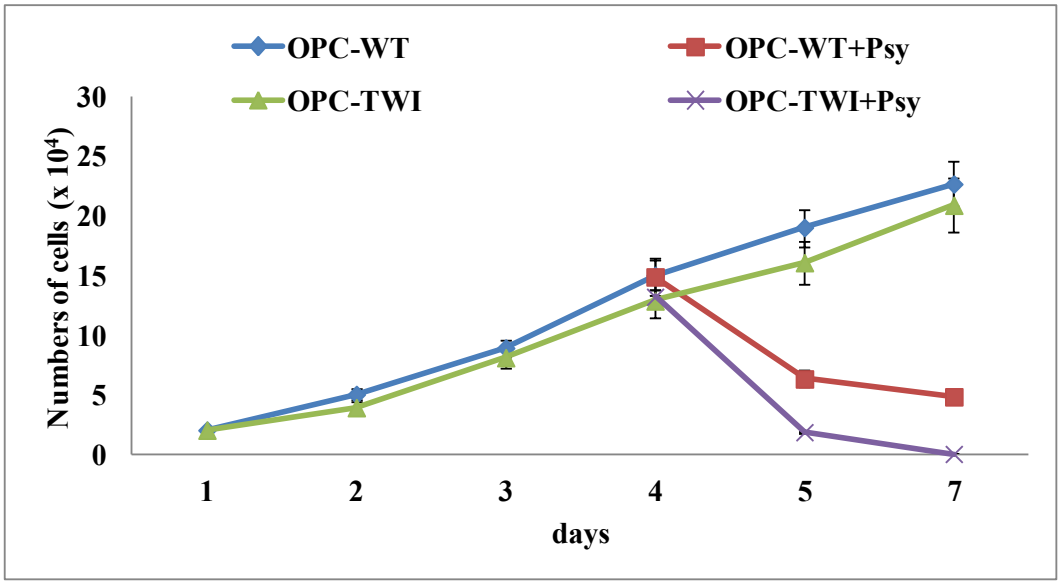

Fig. 23 Growth curves of OPCs
$2x10^4$ cells per well were plated in 24-well culture plate. Cells were trypsinized and counted by using a haemocytometer every day in triplicate. Average of cell countings with S.D. values was calculated statistically. The psychosine exposure (50 μM) was made for 24 h and cell number was counted both after 24 h of treatments and after 48 h of restoring basal growth condition.

As shown in the cell growth curve (Fig. 23), OPC-TWI had a proliferative activity less intense than OPC-WT, in normal condition. After 24 hours exposure to 50 μM of psychosine, both OPCs stopped the proliferation and, when basal culturing conditions were restored, they did not re-establish their proliferative activity. This situation strongly suggests the activation of cell death program.
Significantly, differences were found in psychosine-treated fibroblasts (Fig. 24).

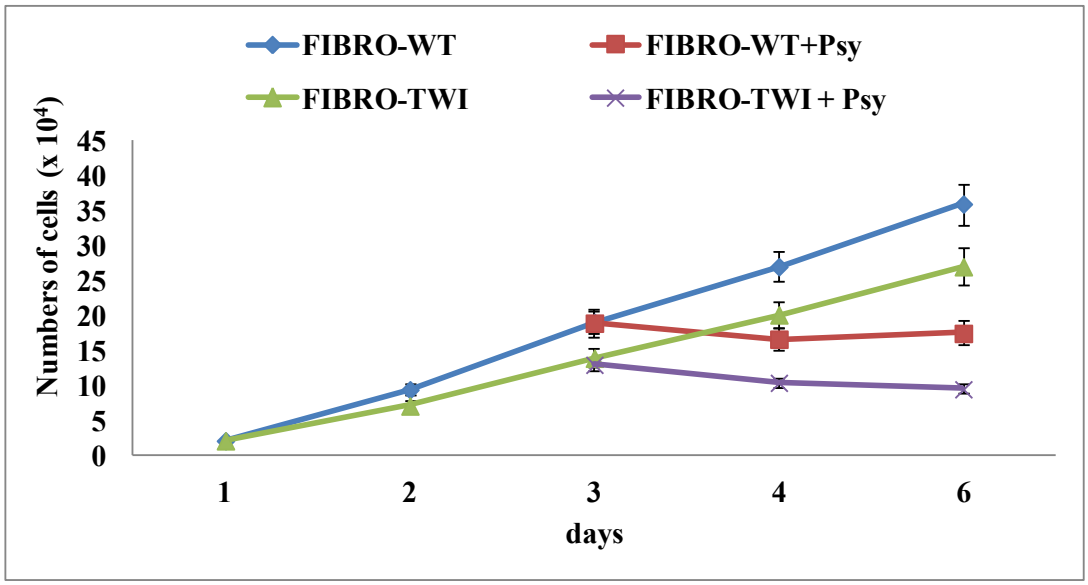

Fig. 24. Growth curves of FIBROs
$2x10^4$ cells per well were plated in 24-well culture plate. Cells were trypsinized and counted by using a Hemocytometer every day in triplicate. Average of cell countings with S.D. value was calculated statistically. The psychosine exposure (50 μM) was made for 24 h and cell number was counted both after 24 h of treatments and after 48h of restoring basal growth condition.

Compared to the untreated cells, an inhibition of the growth curve was detected. After restoring basal culturing medium, FIBROs showed an arrest of replication, that could be reported as a growth-delay. In general, these latter also untreated showed a lower proliferative ability compared to same cells without GALC mutation.

Taken together, these data not only confirmed the different sensitivity of the experimental cells, but also clarified that OPCs viability was drastically compromised by psychosine.

DAPI fluorescent staining was performed for visualizing fragmented-DNA of apoptotic cells and results were reported in Fig. 25. The number of the DAPI stained DNA fragmentation in 1000 random counted nuclei was called Apoptotic Index (AI) in the following description.

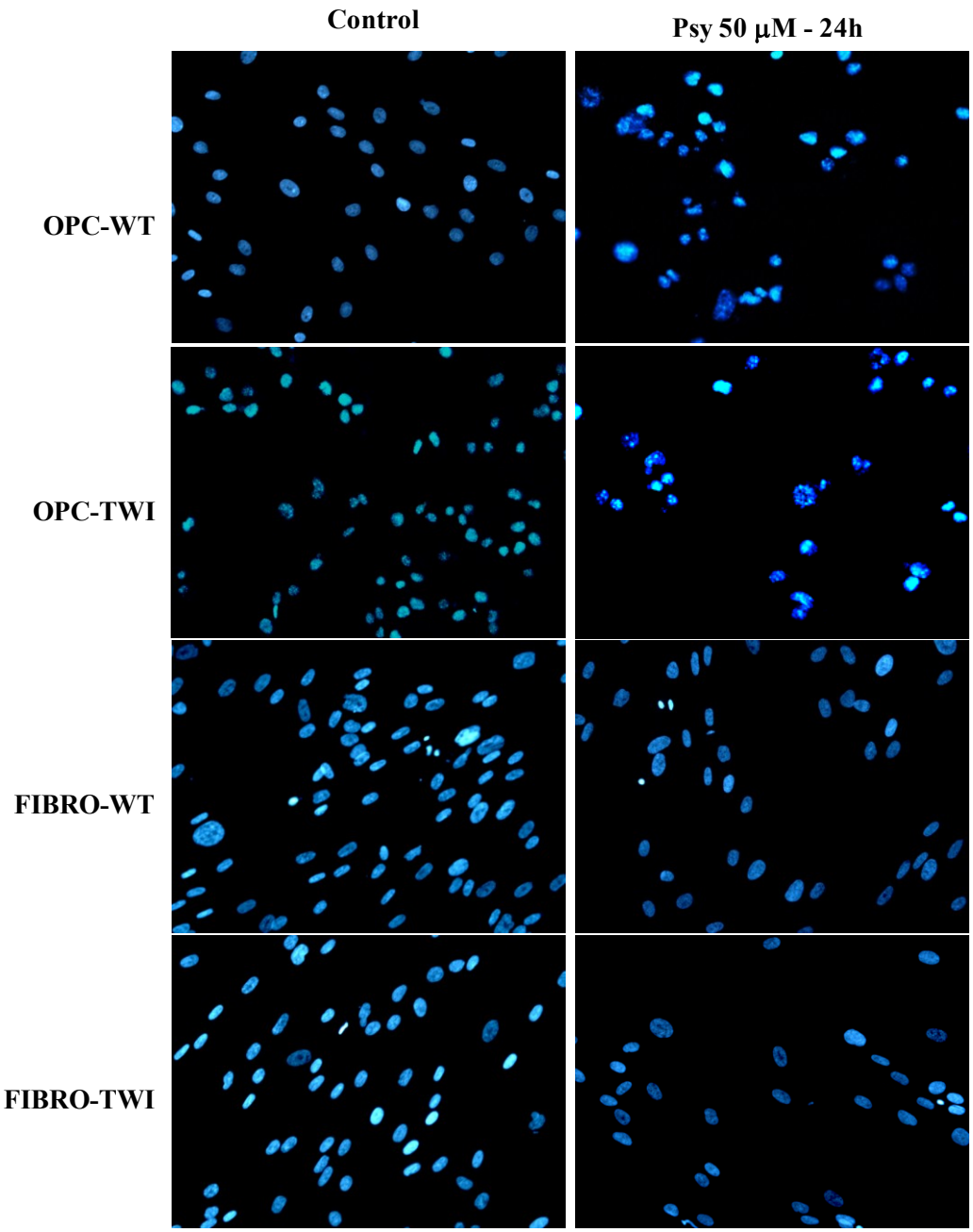

Fig. 25. DNA fragmentation in psychosine treated cells
Cells grown on glass coverslips were exposed to 50 µM of psychosine for 24 h. At the time period indicated, cells were fixed and the DAPI staining analyzed by fluorescent microscopy.

Under control conditions, OPC-WT and FIBRO-WT revealed physiological nuclear morphology, chromatin organization and mitotic events and AI = 0. Low AI was found in both GALC-mutated cells, especially in OPC-TWI (AI= 5±1).

OPCs were found to be very sensitive to exposure to psychosine. At 50 µM psychosine concentration a large number of OPCs responded with apoptotic death, quantified with an AI of 650±18 and 802±43 for OPC-WT and OPC-TWI, respectively (Fig. 25). After fibroblasts treatment, a minor cells density in both lines

was detected and the AI was lower than OPCs ones (FIBRO-WT AI= 180±3; FIBRO-KD AI= 302±4). Thus, cells exhibiting nuclear condensation and DNA fragmentation became detectable in all 50 μM psychosine-treated models. So, the speed of induction of apoptosis by psychosine seemed to be linked to cellular sensitivity at psychosine concentration, while the cellular type or gene profile were not relevant conditions. Under experimental plain, OPCs quickly showed apoptotic phenotype with high AI, while FIBROs showed primary a relevant stop in cell proliferation and an AI significantly augmented versus controls.

Furthermore, even after 24 hours of psychosine exposure a significant cell detachment did not detect in FIBROs, while the OPCs exhibited typical morphological alterations of apoptosis such as rounding and cytoplasmic shrinkage and a significant cell detachment (Fig. 26). The calculation of AI was very complicated in this situation.

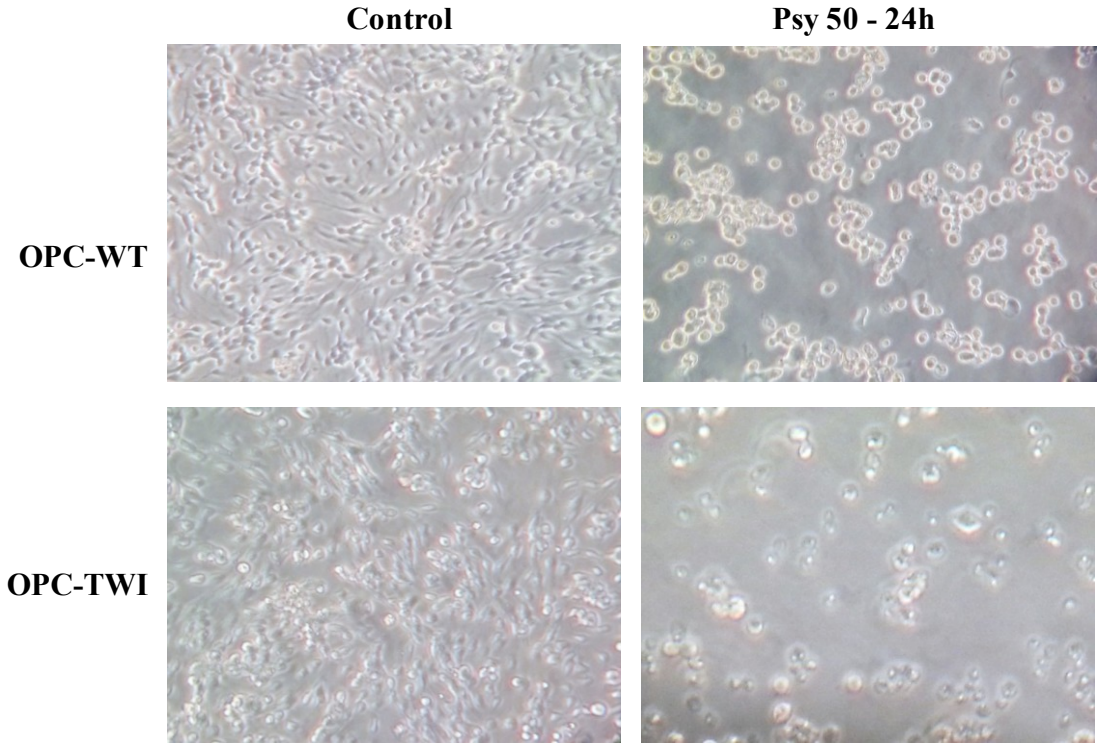

Fig. 26. Morphological changes in psychosine-treated OPCs
OPC-WT and OPC-TWI become round with cytoplasmic shrinkage. Significant cell detachment was observed

DAPI staining of the psychosine treated cells showed the presence of nuclear fragmentation indicating that the cells underwent programmed cell death or apoptosis. To characterize the apoptotic pathway induced by psychosine in our cellular models the activation of caspase-3 was analyzed. Caspase-3 is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the cascade of events associated with apoptosis. Activated caspase-3 is responsible for the breakdown of several cellular components related to DNA-repair and

regulation, such as poly ADP-ribose polymerase (PARP) (Schlegel et al. 1996), and nulear laminins (Lazebnik et al. 1995). Cells were treated with 20 µM and 50 µM of psychosine for 24 h and the analysis of caspase-3 activation was made by colorimetric assay and western blot analysis, also.

In Sandwich ELISA colorimetric assay, the level of caspase active form in the cell lysate is directly proportional intensity of colour (Fig.. 27).

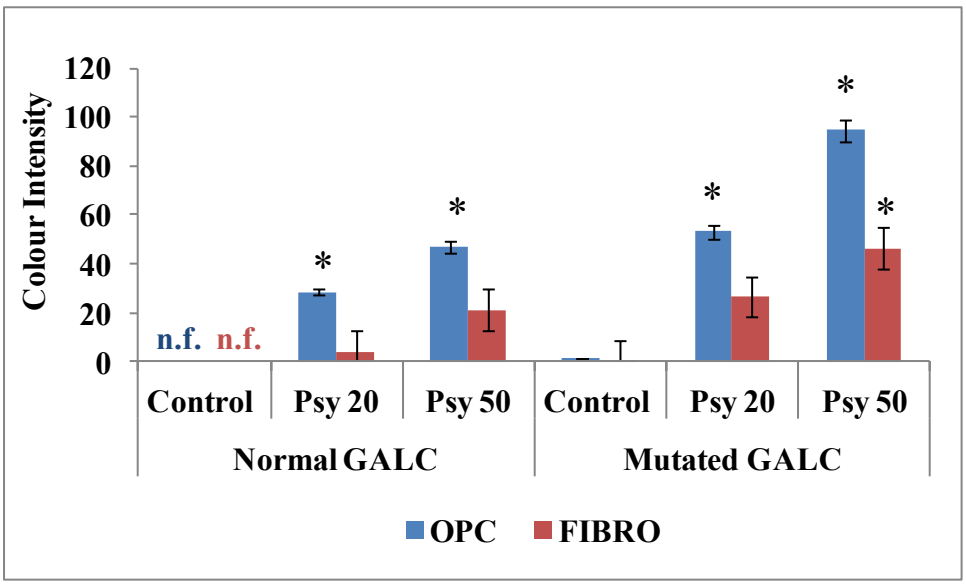

Fig. 27. Sandwich ELISA for caspase-3 activation in cell models treated with psychosine
Cell lysate were tested and quantified spectrophotometrically at the wavelength of 450 nm. Each colon represents mean ± SD (n = 4) *P< 0,05.

It was revealed a deep yellow coloration in treated OPCs, especially in OPC-TWI. Indeed, in the fibroblast lines the caspase-3 active form was lower detected, as revealed by color intensity.

Due to some problem for ELISA sensibility, it was important to confirm whether caspase 3 was activated by proteolysis of the inactive pro-enzyme. So, the expression of caspase-3 - active form protein - was analyzed by western blot analysis, also. To detect the dose-response, two concentrations of psychosine were applied in all cells and time incubation set at 24 hours (Fig. 28).

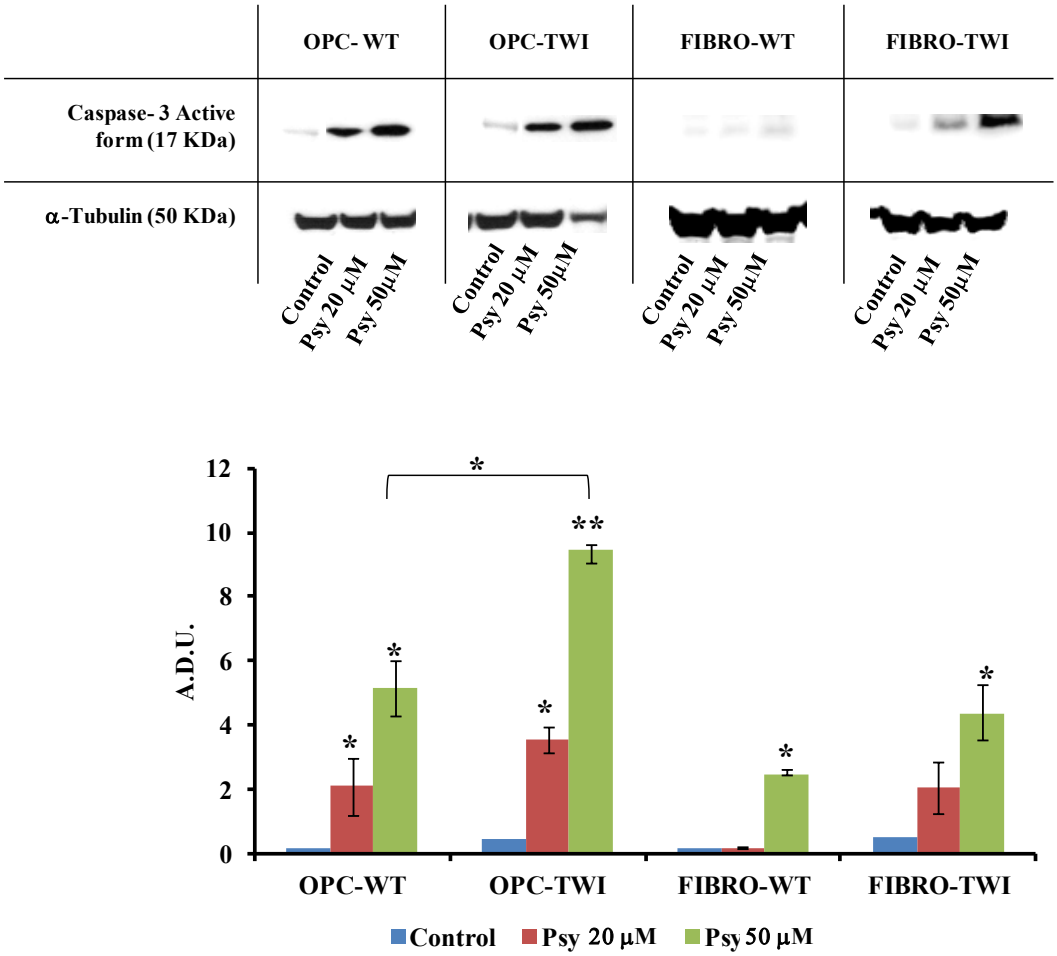

Fig. 28. Active caspase-3 expression in cell models treated with psychosine
Cells were lysate and level of searched protein was quantified by immunoblotting. Each colon represents mean of ratio specific protein/standard protein ± SD (n = 3) *P< 0,05; **P<0,01

As shown in Fig. 28, the levels of Caspase-3 active enzyme increased in response to 50 µM psychosine with a cellular sensitivity could be schematized in this way: FIBRO-WT < FIBRO-TWI < OPC-WT < OPC-TWI, according to our preliminary data from MTT test. More information could be obtained from 20 µM psychosine-treatment. OPC-WT and FIBRO-TWI showed similar Caspase-3 level, while OPC-TWI enhanced it. In contrast, caspase-3 levels in treated FIBRO-WT were similar to those of their own control.

These data suggested that the apoptosis psychosine-activated was not GALC mutation-dependent, because high concentration of lipid determined caspase activation in all models. Indeed, a relationship between concentration and damage induction could be established, as well as the molecular mechanism involved in cell sensitivity must be examined.

Considering the knowledge obtained from the cell growth curve, the phosphatidylinositol-3-kinase PI3K pathway was investigated. Generally, PI3K

pathway plays a central role in cell survival, as suggested by the observation that PI3K inhibitors are known for block of it. PI3K activity is finely regulated by Phosphatase and Tensin homolog (PTEN) that is report as a tumor suppressor factor. So, PI3K and PTEN expression were detected in our cellular models (Fig. 29)

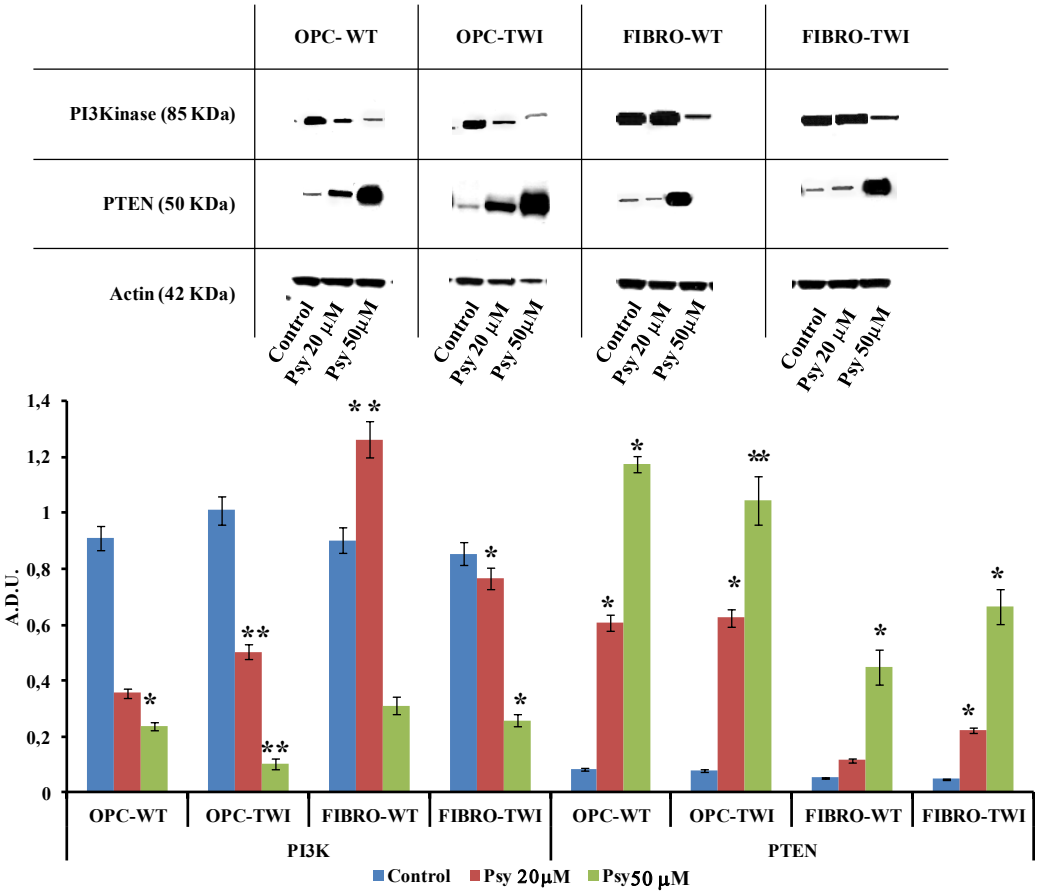

Fig. 29. PI3K and PTEN expression in cell models treated with psychosine
Cells were lysate and level of searched protein was quantified by immunoblotting. Each colon represents mean of ratio specific protein/standard protein ± SD (n = 3) *P< 0,05; **P<0,01

Western blotting of total cell extracts shows a single band for PI3K and another single band for PTEN with an apparent molecular weight of 85 and 50KDa, respectively. PI3K band was detected in all condition and showed a general decrease in treated-cells proportioned to psychosine dose. One exception was found for FIBRO-WT treated with 20 µM of psychosine. In contrast, the PTEN levels were abundantly dose-dependently up-regulated. The same data were plotted as percentage of protein expression for each cell type compared to respective untreated control. In this case, the control value was reported as 100 and this value was subtracted for obtaining the results reported graphs (Fig. 30)

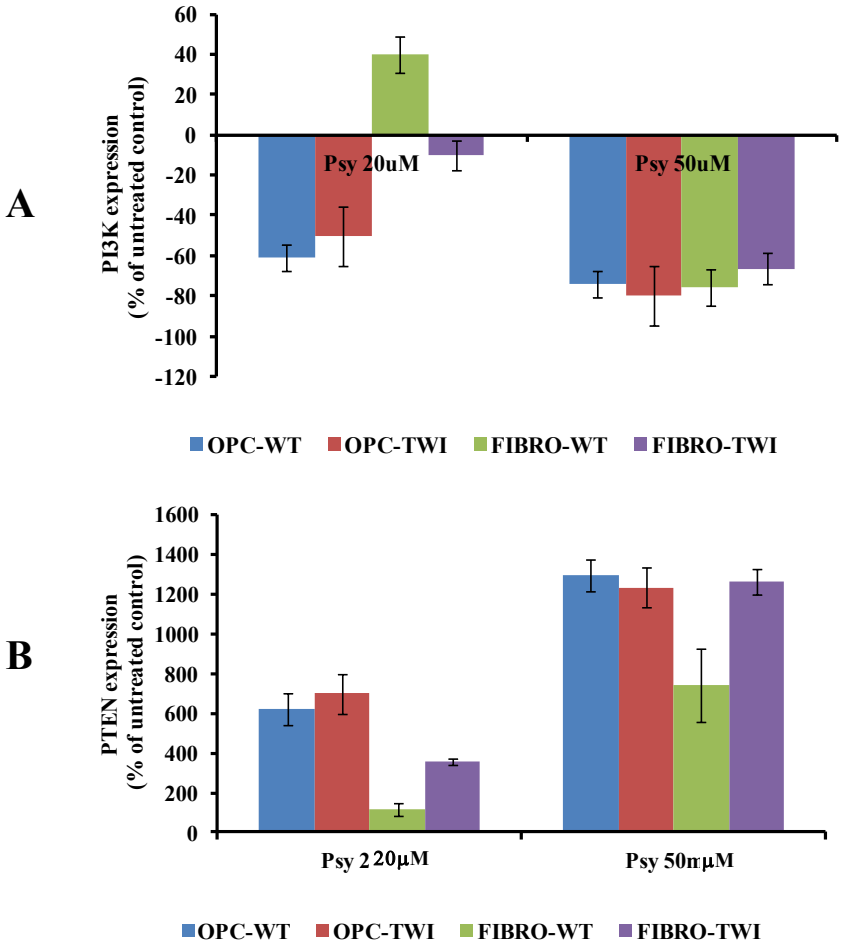

Fig. 30. Percentage of PI3K (A) and PTEN (B) expression on psychosine-treated cell models compared to respective untreated control.
This data derived from the relative expression showed by Western Blot analysis

The results underlined more strongly the down-expression of PI3K and the inverse tendency of PTEN in cellular response linked to psychosine concentration. Moreover, FIBRO-WT incubated with 20 µM showed a residual PI3K expression that is not detected at highest concentration tested. This data might the differences that cell growth curve and MTT reported.

Thus, the psychosine-mediated survival collapse seemed due to disturbance of membrane-activated PI3K. For evaluating kinase activity, some of its targets were investigated. First of all, the pro-apoptotic member of the Bcl-2 family, Bad (Fig. 31).

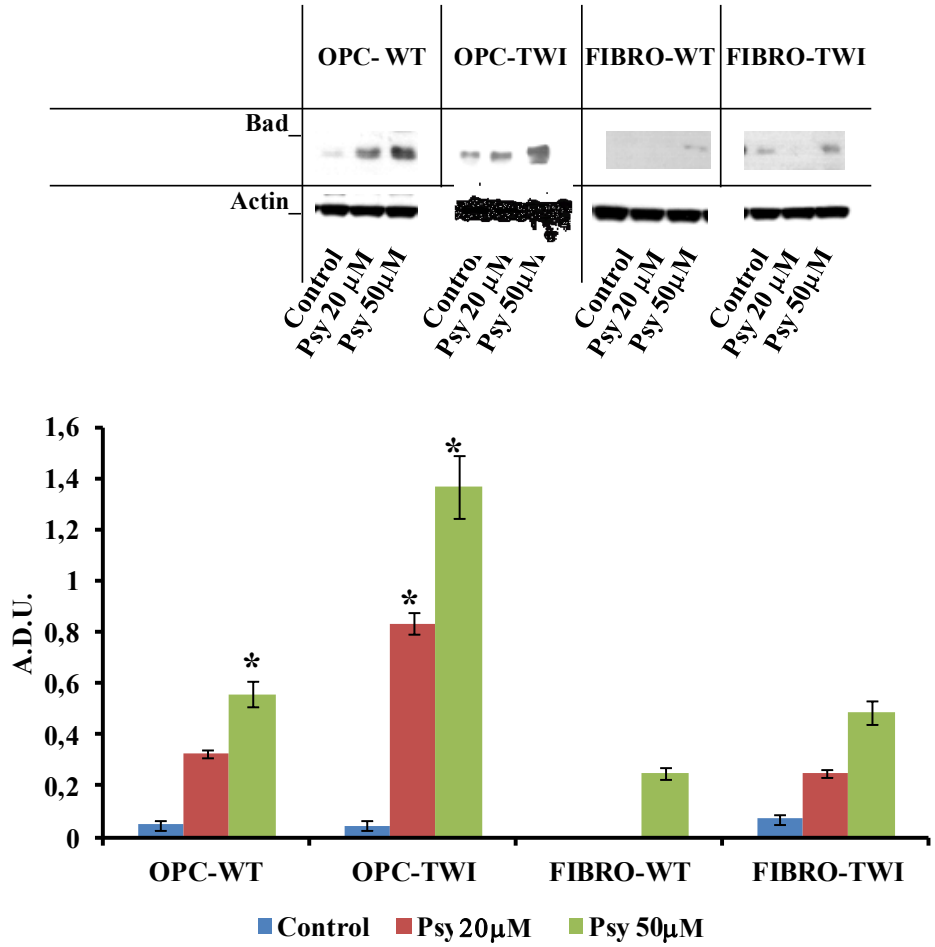

Fig. 31. Unphosphorilated Bad level in cell models treated with psychosine
Cells were lysate and level of searched protein was quantified by immunoblotting. Each colon represents mean of ratio specific protein/standard protein ± SD (n = 3) *P< 0,05.

Fig. 31 shows the expression of unphosphorilated Bad in our cellular models. OPCs reported a dose-dependent response more intense than FIBRO-TWI. At highest concentration the free citoplasmatic Bad was three times more present in OPC-TWI than in FIBRO with GALC mutated, while it was detected in 50 µM psychosine-treated FIBRO-WT.

Moreover, PI3K-dependent transcription factors were investigated p53 and NF-kB, which are known as regulator of proapoptotic and antiapoptotic genes, respectively. The results of p53 and NF-kB immunoblotting were reported in Fig. 32 and Fig. 33, respectively.

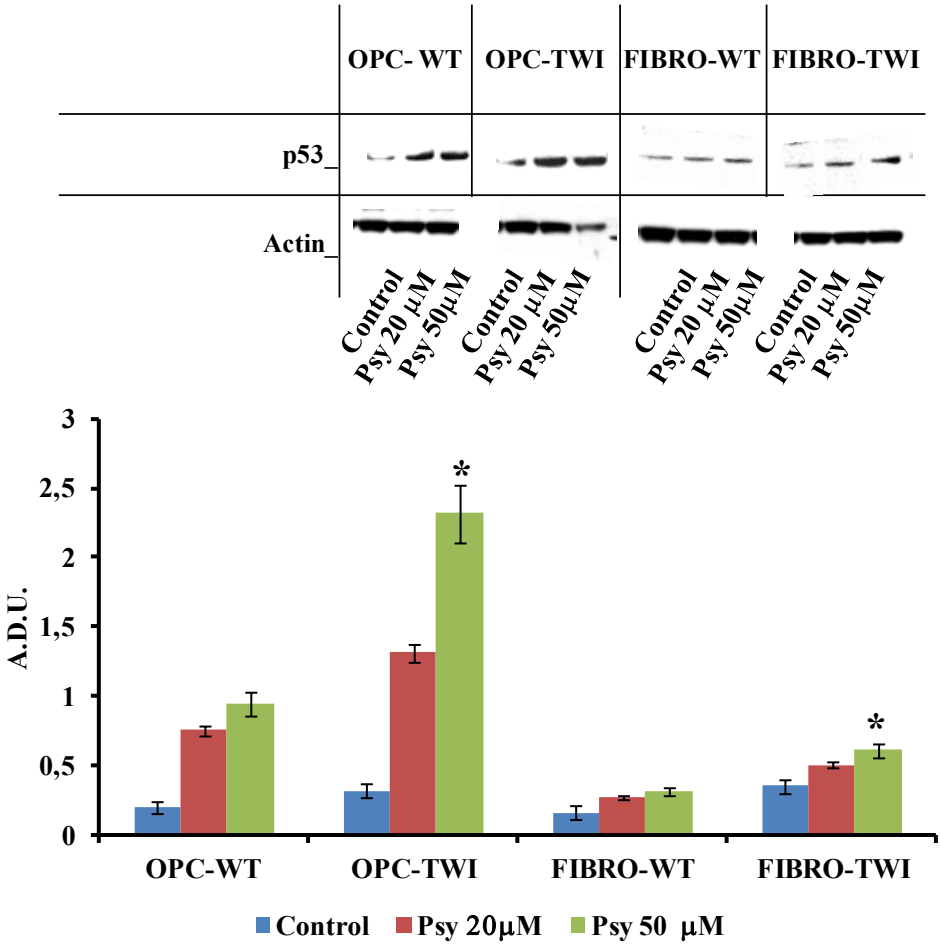

Fig. 32. p53 expression in cell models treated with psychosine
Cells were lysate and level of searched protein was quantified by immunoblotting. Each colon represents mean of ratio specific protein/standard protein ± SD (n = 3) *P< 0,05.

As consequence of psychosine action, the p53 values increased with a dose-dependent cell response and, as expected, with a FIBRO-WT < FIBRO-TWI < OPC-WT < OPC-TWI cell reactivity.

Since activation of NF-κB could be regulated by PI3K activity and considering that its activity regulates expression of protein involved in cell survival, NF-kB status was examined in response to psychosine (Fig. 33).

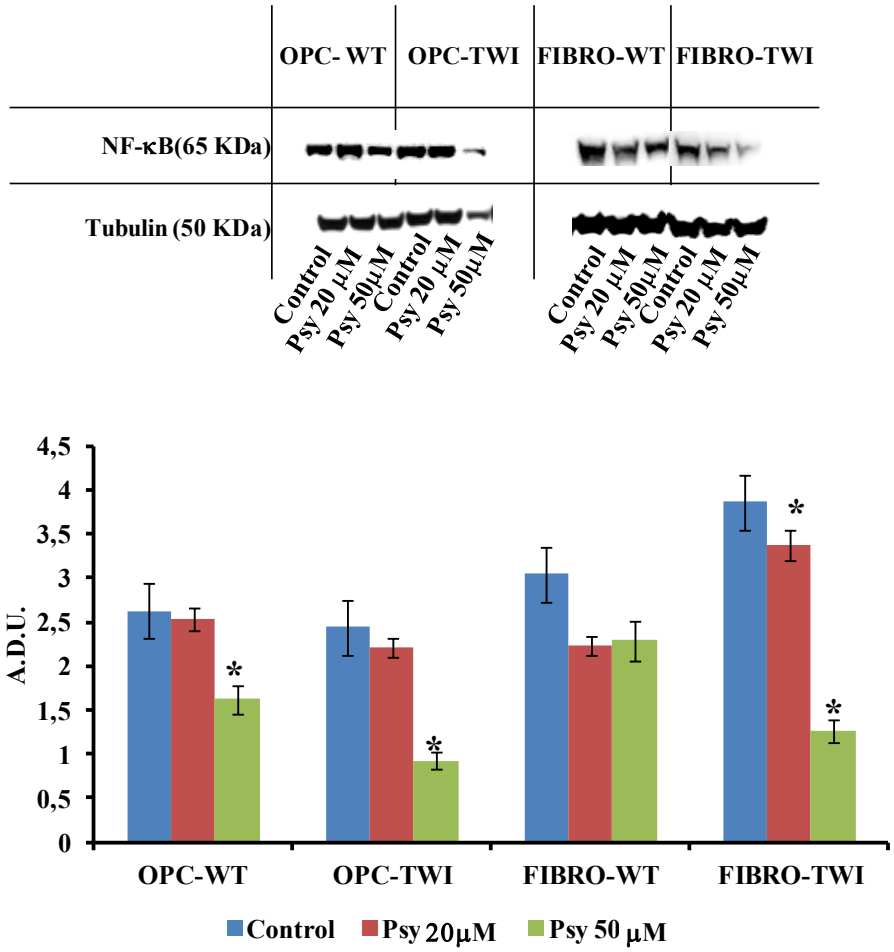

Fig. 33. NF-κB (p65) immunoblot in cell models treated with psychosine
Cells were lysate and level of searched protein was quantified by immunoblotting.
Each colon represents mean of ratio specific protein/standard protein ± SD (n = 3) *P< 0,05

In our cellular model was found a decrease of free NF-kB in total cell lysate, and the percentages of decrease respect to basal control were 3,6±0,29; 9,2±0,18; 26,8±0,8; 12,6±0,6 for 20 μM treatment, while 38,1±0,7; 2,14±0,9; 24,7±0,34 and 67,3± 0,8 for 50 μM in OPC-WT, OPC-TWI, FIBRO-WT and FIBRO.TWI, respectively.

Taken together, the results suggest psychosine affected cell survival thought deprivation of survival signalling that interact on cellular membrane. This event was not cell specific, but dose-dependent. OPCs quickly activated the apoptosis program, even if OPC-TWI were the highest sensitive, probably due to higher psychosine level picked in. FIBRO-WT showed more resistance at psychosine-inducted apoptosis at lower concentration tested because the survival pathway was still able to interact in cellular membrane, while FIBRO-TWI did not report the same capability.

Other membrane-linked proteins were analyzed: the connexins that play essential roles in cell homeostasis, growth, differentiation and death. Among connexin family members, Connexin43 is an active element in apoptosis by Gap junction-dependent mechanism or gap junction-independent effects that could be mediated through hemichannels or through Connexin 43 per se. Still today, there are no scientific researches evaluating Cx43 expression and localization in normal and in GALC mutated oligodendrocytes precursor cells.
Thus, we analyzed Cx43 expression in our cellular models with and without psychosine treatment (Fig. 34).

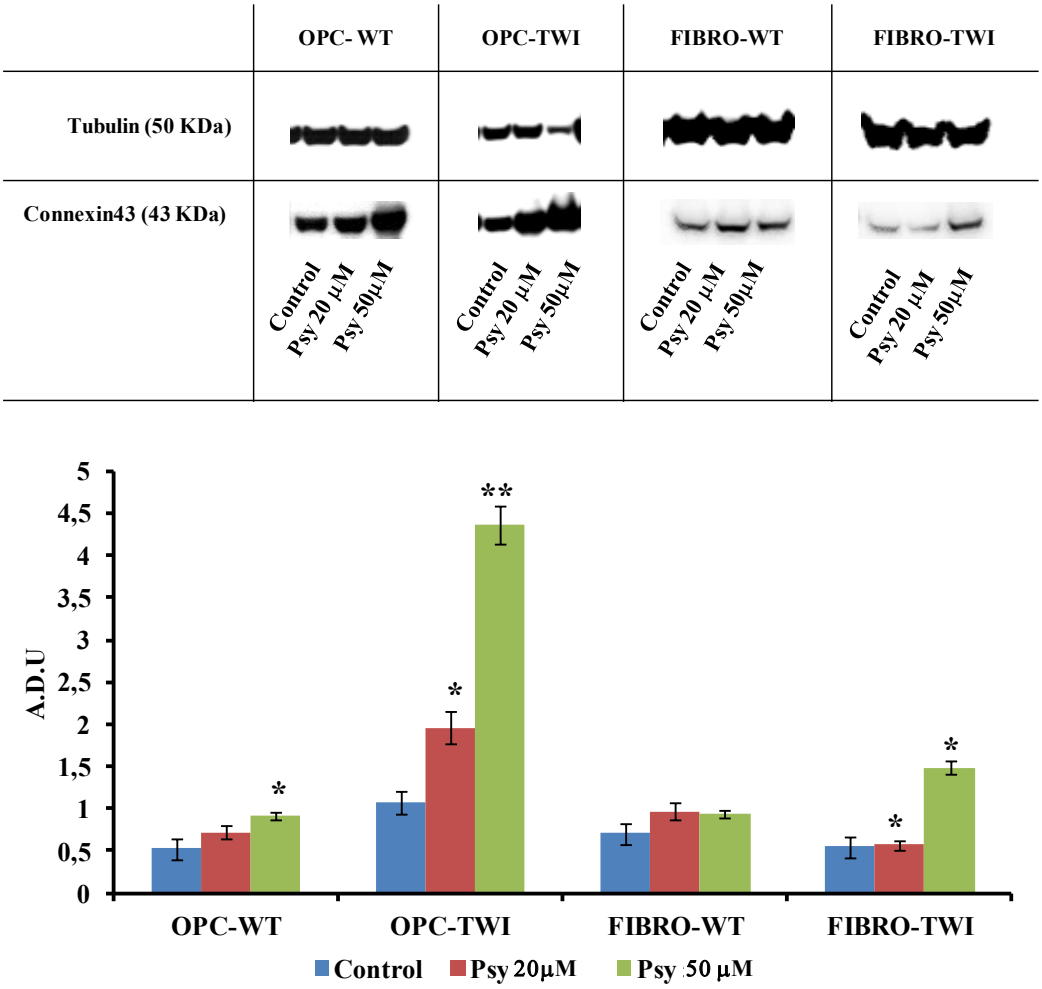

Fig. 34. Connexin43 immunoblot in cell models treated with psychosine
Cells were lysate and level of searched protein was quantified by immunoblotting.
Each colon represents mean of ratio specific protein/standard protein ± SD (n = 3) *P< 0,05

As shown in Fig. 34, exposure of the OPC-TWI to psychosine for 24 h lead to a rapid increase in Cx43 expression, gradually augmented with increasing concentration of lipid (from 20 to 50 µM). The lower dose tested never induced a significantly Cx43 increase in OPC-WT and in FIBRO-TWI, while at higher concentration of

psychosine Cx43 levels were amplified. FIBRO-WT showed the same Cx43 intensity with both treatments. In addiction, analysing immunoblotting of controls it was found that OPC- TWI had a major basal expression despite OPC-WT, while in FIBROs the basal condition were opposed: this could be useful for understanding the human situation.

The effect of psychosine upon Cx43 expression was evaluated in OPCs at high cell density (70000 cells/ml) and low cell density (500 cells/ml) in order to define if cell-cell contacts are required. Results obtained were the same, and the density-independence of psychosine action in enhancer Cx43 levels suggested that Cx43 is involved in psychosine-inducted apoptosis with a gap-junction independent function.

Immunofluorescence staining assay was applied to examine where Cx43 was localized in control and treated OPCs (Fig. 35).

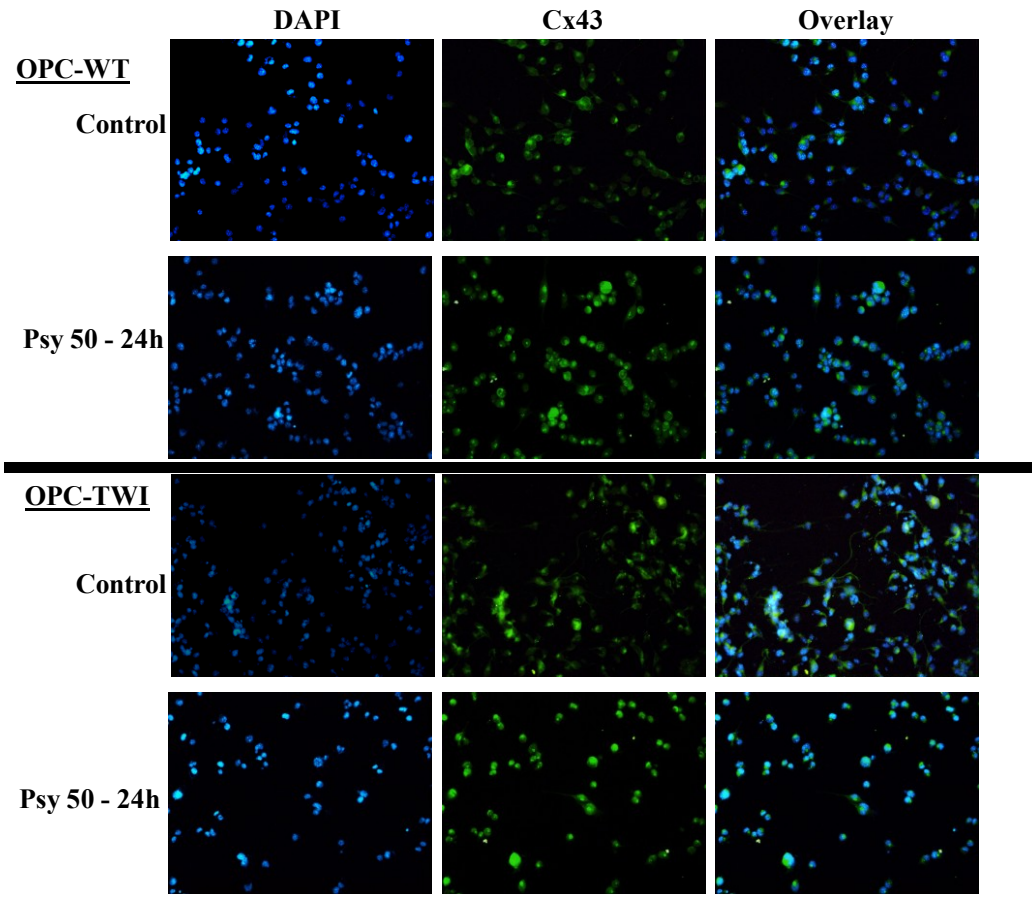

Fig. 35. Immunocytochemistry of Cx43 in OPCs

As shown in Fig. 35, immunoreactivity for C terminal region of Cx43 was observed in cytoplasm of control cells, with particular intensity in regions corresponding to Golgi apparatus around the nucleus. After 24 h of 50 μM psychosine treatment, Cx43 never change its localization, but it is organized in clusters and some punctuated fluorescent slicks are detected into the nucleus, also.

In order to confirm *in vivo* the Cx43 augmented expression, brains lysate from wild-type and *twitcher* mice were immunoblotted for Cx43. Two times were analyzed: postnatal day 20 and postnatal day 40, corresponding to symptoms appearance and moribund status for *twitcher* mouse, respectively (Fig. 36).

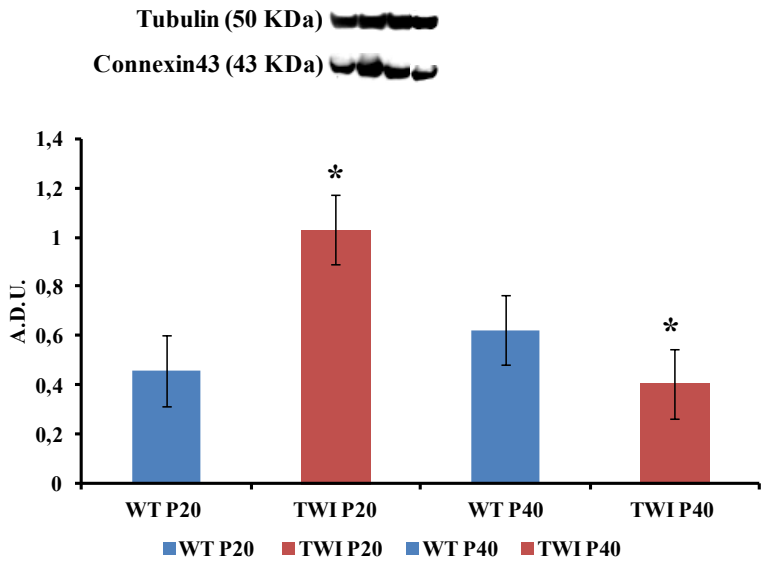

Fig. 36 Cx43 expression in mice brains *P<0,05 (n=7)

At symptoms appearance, *twitcher* mice showed a twofold higher expression of Cx43 respect to wild-type, while in period of occurring death the protein levels were detected less intensely despite wild-type mice. This last event could be explained by the oligodendrocytes loss and the activation of a strongly astrocytes-mediated innate immune response to cellular RNA that is released as a result of tissue damage (Zhao et al, 2006).

As only a speculative pleasure, astrocytes obtained from human glioma were characterized as $GFAP^+$ and exposed to a not cytotoxic dose of psychosine (20 μM). Results showed a decrease in Cx43 levels with no change in cell viability and morphology (Fig. 37).

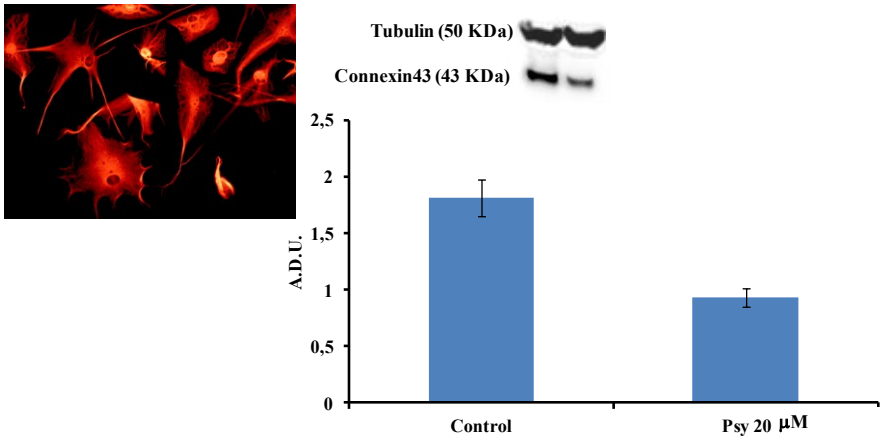

Fig. 37. GFAP-positive cells obtained from human glioma were treated with psychosine and cell lysate analyzed for Cx43 expression.

This isolated data confirmed our hypothesis that cell sensitivity to the accumulated-lipid-accumulated in KD pathogenesis is linked to Cx43 related expression in cell response, because the key mechanism of action remains the same.

Considering this new advance Connexin43 expression was monitored during wild-type- and GALC-mutated oligodendrocytes maturation steps without psychosine treatment.

Differentiation of these cell lines was a difficult task. Primary oligodendrocytes undergo differentiation in defined medium containing low serum concentration, but this condition was not sufficient for the OPCs stabilized lines. Modification of common differentiating media, by changing the concentrations and combinations of their components, was not sufficient to induce differentiation of OPCs culture; moreover poor survival of the cells was observed.

Based on the unsatisfactory results obtained, a careful evaluation of the culture conditions of the progenitor cells was made. To induce proliferation and to prevent the maturation of oligodendrocyte progenitors are cultured in medium containing growth factors, such us bFGF and PDGF-AA (Saneto et al., 1985). For inducing differentiation, cells were private of growth factor and the medium was further supplemented with D-galactose, for giving to cells a higher accessibility of carbohydrate source, and with B27, a medium supplement known to increase neuronal survival in primary CNS cultures (Sheng et al., 1989), and for enhancing oligodendrocytes survival (Yang et al., 2005).
In this medium OPC-WT differentiated and survived significantly up to 5 days. At 72 h after differentiation induction, OPC-WT showed an extensive network of processes and membranes typical of well differentiated myelinating cells (Fig. 38).

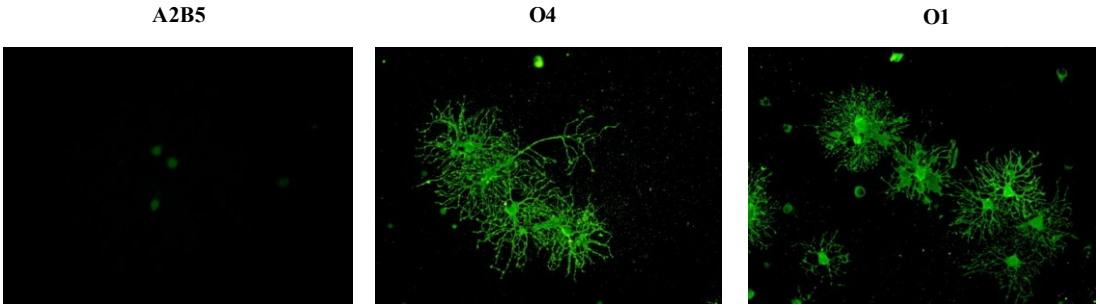

Fig. 38. Immunofluorescence of differentiated OPC-WT

In this medium OPC-WT were properly differentiated in a well homogenous population, in which A2B5 positive cell did not detected, while in cellular surface O4 and O1 was found.

The same medium was applied to OPC-TWI, but markers of differentiation were not immunostained. Indeed, a great number of A2B5 and Cx43 positive cells were found (Fig. 39A).

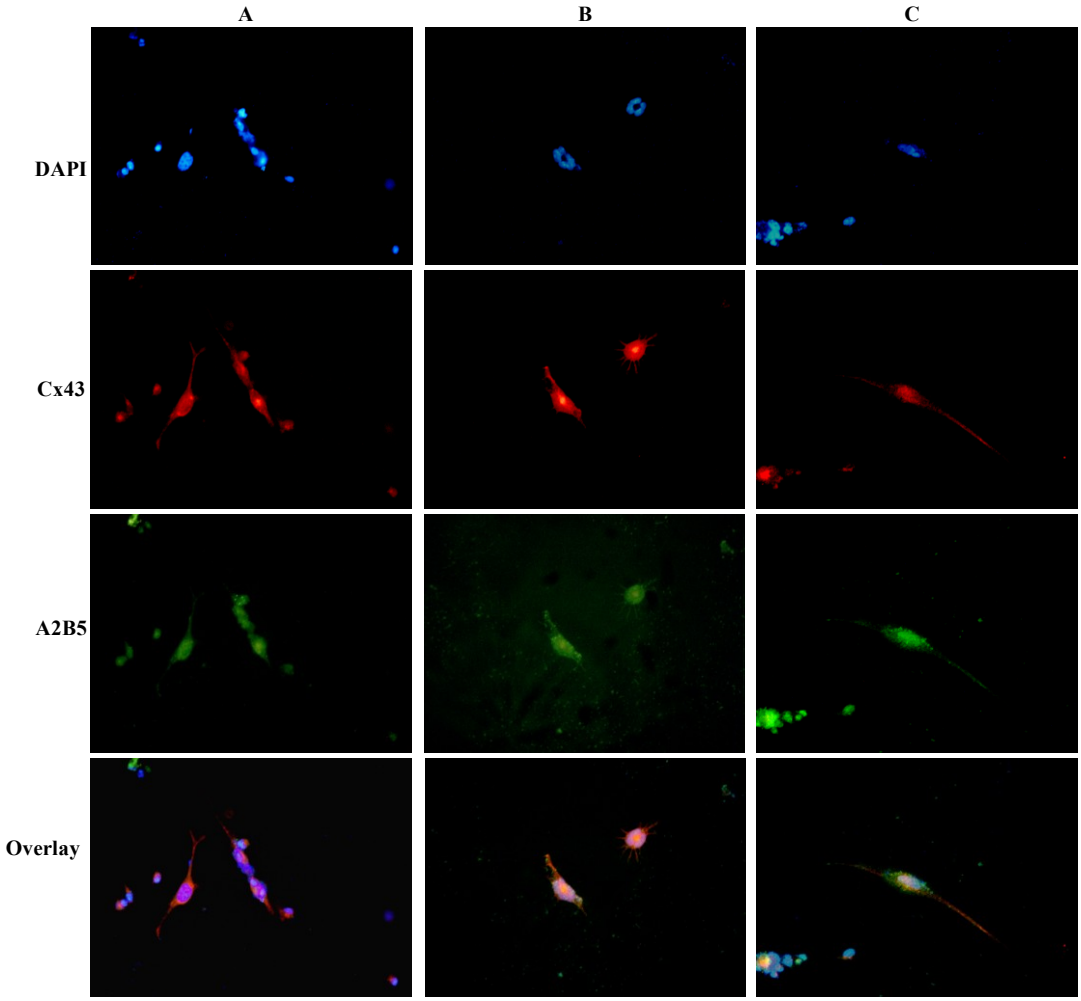

Fig. 39. OPC-TWI immunostained in differentiating medium

In addition, Fig. 39B and C reported the appearance of multinuclear cells, with an aberrant morphology and expression of the C-terminal domain of Cx43. This event suggested that probably psychosine was accumulating in this cell, even if no caspase-3 activation was found.

Taken together, these findings led to the general idea that psychosine may functionally impact cells through interference on the growth system and alteration in maturation programme, strongly related to Connexin 43 expression. Thus, the likelihood of using adipose tissue-derived mesenchymal stem cells (AT-MSCs) as source of oligodendrocytes became more and more attractive.

At first, AT-MSCs were identified eith immunocytochemical and flow cytometry procedures for several cell surface markers.
In the immunocytochemistry analysis performed after the first passage, MSCs did not show labeling for the hematopoietic line, tested by antibodies for CD14, CD34 and CD45. On the contrary, they were 10 positive for the adhesion molecules specific for MSCs, such as CD44 (H-CAM), CD90 (Thy 1), and CD105 (Endoglin) (Fig. 40).

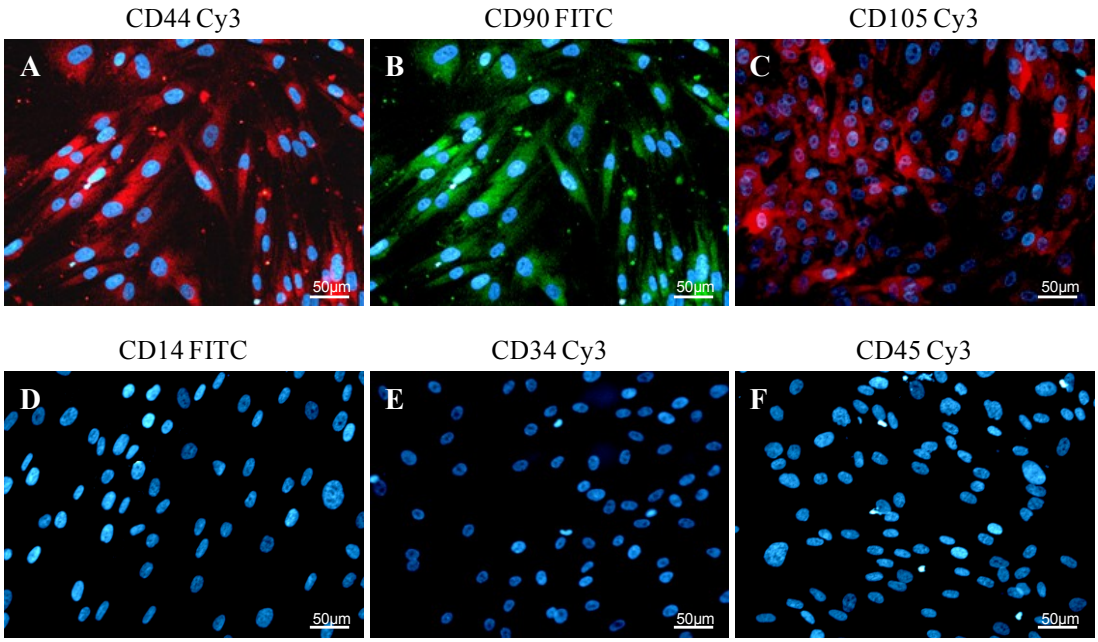

Fig. 40. Immunophenotypical characterization.
CD44 (A), CD90 (B), CD105 (C) (positive markers), CD14 (D), CD34 (E), and CD45 (F) (negative markers) mesenchimal stem cells (MSCs) markers expression by immunocytochemistry and using a Leica DMRB fluorescence microscope equipped with a computer assisted Nikon digital camera. Panels A-F: Magnification x40; Scale bars: 50 µm. DAPI stained MSCs were superimposed to show cell nuclei.

Flow cytometry results confirmed that cells were positive for CD44, CD90 and CD105, and negative for CD14, CD34 and CD45 (Fig. 41).

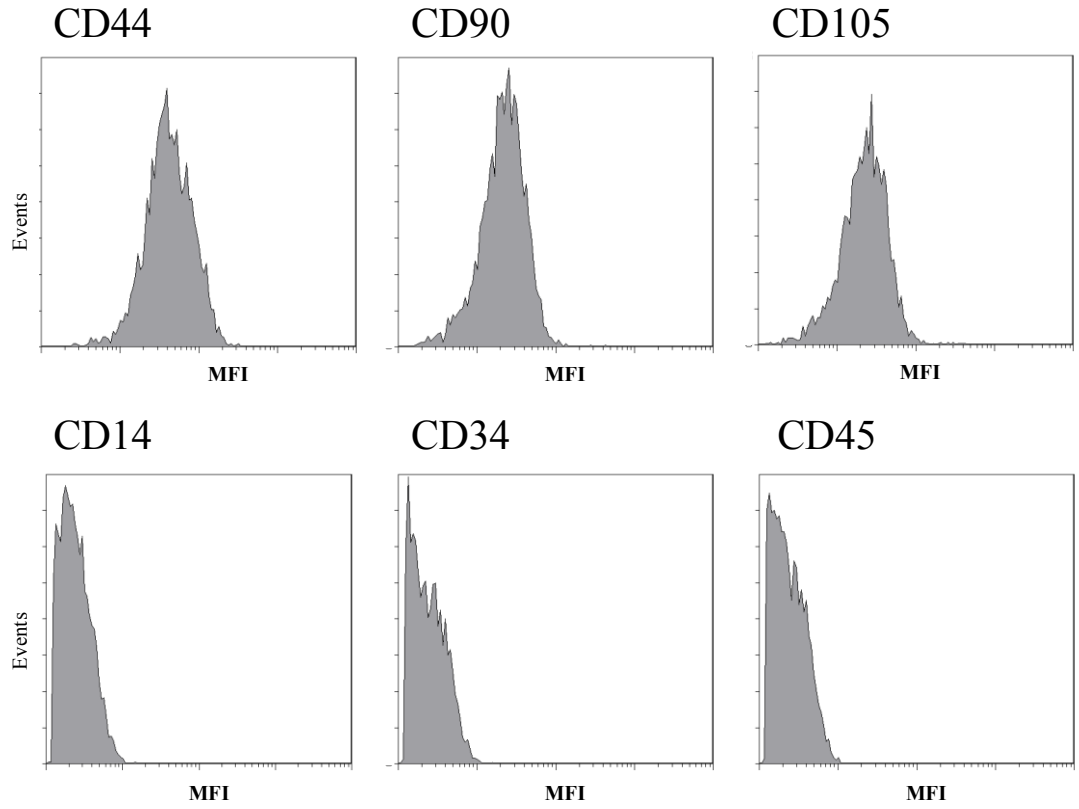

Fig. 41. CD44, CD90, CD105 (positive markers), CD14, CD34, and CD45 (negative markers) mesenchimal stem cells (MSCs) markers expression by flow cytometry.
A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at λ= 488 nm and fluorescence was monitored at λ= 525 nm. Fluorescence was detected using logarithmic amplification. Mean fluorescence intensity (MFI) values were calculated and recorded automatically.

Two different conditioned media were applied and cell morphology of control, OECs-CM or B104-CM treated AT-MSCs was analyzed and some markers indicating their potential differentiation in neurons or glial cells were tested by immunostaining and flow cytometry. In particular, the expression of nestin, protein gene product 9.5 (PGP 9.5), microtubule-associated protein 2 (MAP2), glial fibrillary acidic protein (GFAP) and neuron cell surface antigen (A2B5) induced by OECs-CM or B104-CM was examined at 24 hours and 7 days of culture.

The morphology of control cells appeared quite similar at 24 hours and 7 days, whereas marked differences were observed using conditioned media and at longer period of culture (Fig. 42 A and B, respectively). Data indicating smallest size and lowest complexity, close to the origin of diagrams, were likely to be referred to debris and were not considered.
At 24 hours, MSCs cultured in control medium demonstrated typical stromal cell morphology with a characteristic spindle-like shape with elongated projections. At this time, control cells appeared of large dimension and not very complex in shape,

while the cells treated with OECs-CM were of smaller size, more elongated and complex. Cells treated with B104-CM were similar to the control (Fig. 42A). Following 7 days, the number of control AT-MSCs cells was increased, but their morphology was similar to cells at 24 hours. At 7 days the morphology of cells treated with either OEC-CM or B104-CM were of smaller size and with greater complexity. A reduction of number of cell was also 11 detected (Fig. 42B). Exposition to OECs-CM or B104-CM induced marked differences. Cells were significantly fewer and smaller, and feature a much higher complexity.

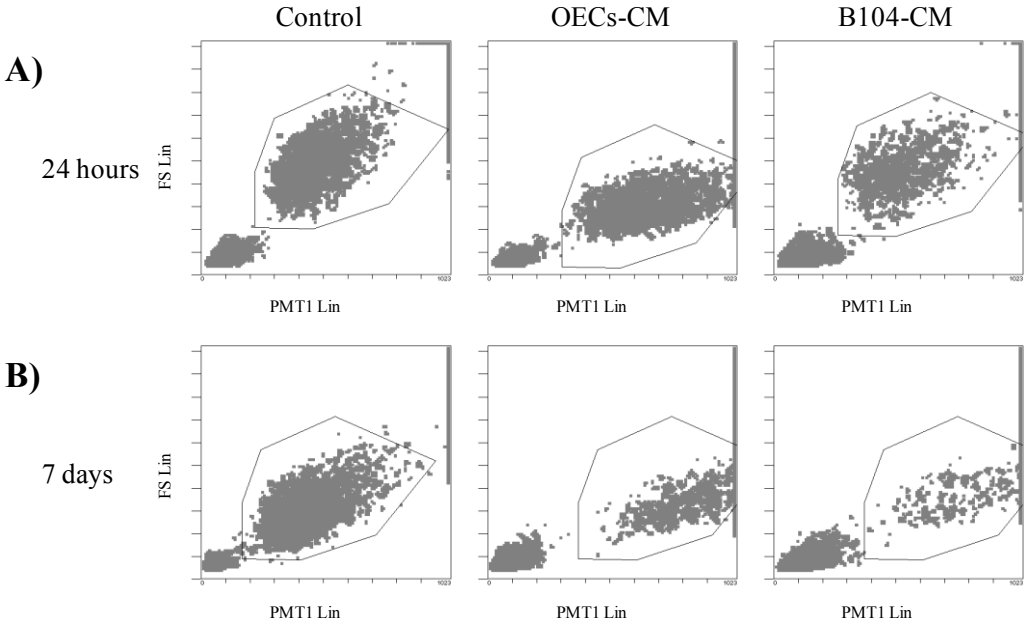

Fig. 42. AT-MSCs morphology analysis of control and OECs-CM or B104-CM treated cells by flow cytometry 24 hours (A) and 7 days (B) after the treatment.

At 24 hours, MSCs cultured in control medium were clearly immunopositive for nestin and PGP 9.5, at a more low level for MAP2, but very low fluorescence was detected for GFAP and A2B5 (Fig. 43). Compared to control MSCs, cells cultured in OECs-CM or B104-CM appeared more positive for nestin and PGP 9.5, more weakly stained for MAP2 and very few cells were immunopositive for GFAP and A2B5 (Fig. 43).

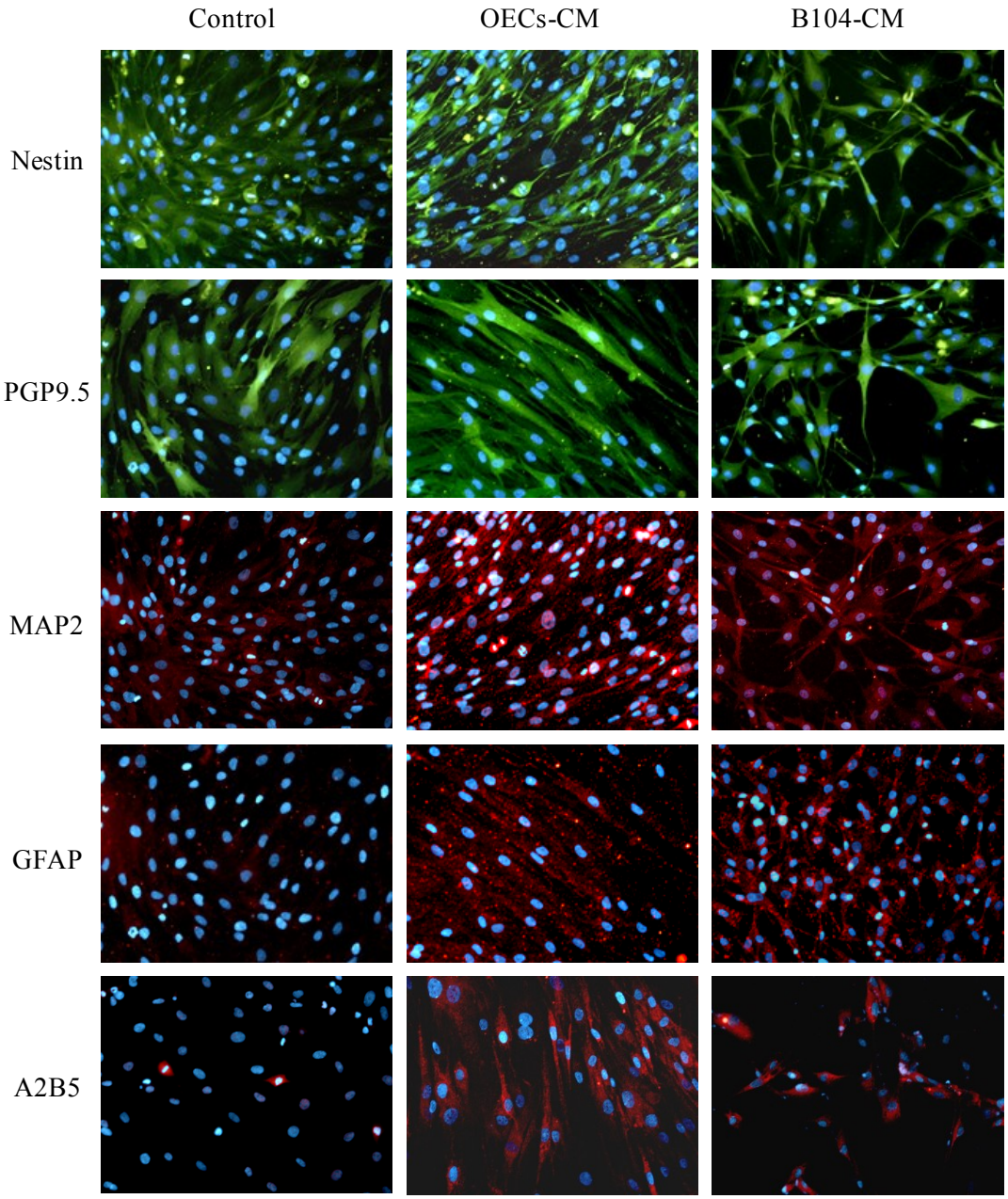

Fig. 43. Expression of differentiation markers by immunocytochemistry of control and OECs-CM or B104-CM treated AT-MSCs 24 hours after the treatment.

Following 7 days, exposition to OECs-CM or B104-CM induced marked differences (Fig. 44).Immunostaining for all markers, especially for PGP 9.5, was more pronounced with respect to controls and to correspondent cultures at 24 hours.

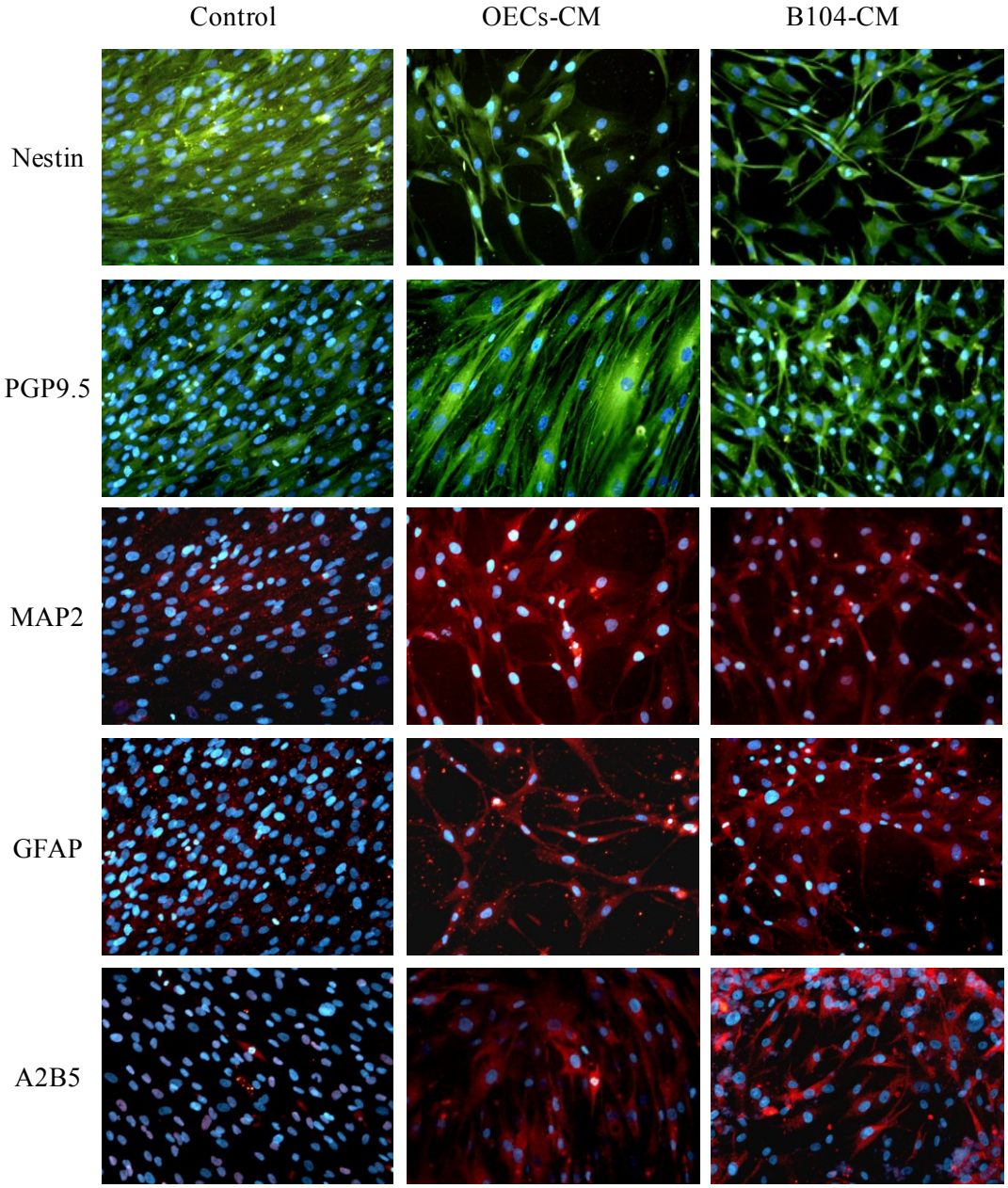

Fig. 44. Expression of differentiation markers by immunocytochemistry of control and OECs-CM or B104-CM treated AT-MSCs 7 days after the treatment

On the whole, data obtained by flow cytometry were in keeping with observations at the light microscope for immunostaining experiments.
The expression of experimental markers was evaluated by mean fluorescence intensity (MFI) and percentage of positive cells. Results obtained for the different markers, using different media at each time point were summarized in Figs. 45-49 and Table 6.

		Control		OECs-CM		B104-CM	
		24 hours	7 days	24 hours	7 days	24 hours	7 days
Nestin	MFI	6.1	12.2	8.0	25.0	7.5	28.4
	% positive cells	97	97	97	98	98	98
PGP9.5	MFI	19.4	18.9	28.1	52.0	27.0	31.7
	% positive cells	99	98	99	98	99.63	98.23
MAP2	MFI	2.5	2.2	3.3	5.0	2.5	3.3
	% positive cells	96	94	98	97	96	94
GFAP	MFI	1.8	1.3	3.0	1.8	2.0	1.9
	% positive cells	66	23	88	80	66	50
A2B5	MFI	1.7	1.2	1.7	2.0	2.8	1.4
	% positive cells	6	25	8	73	6	59

Table 6. Effects of OECs- CM or B104-CM on AT-MSCs determined by Flow Cytometry

- Nestin

At 24 hours, percentage of positive cells for nestin was considerably high either in control cultures or using conditioned media (above 90%). It was still high at 7 days. MFI was similar at 24 hours in all cultures with values slightly higher in those OECs-CM or B104-CM treated, and increased at 7 days in all samples, especially in cells treated with OECs-CM or B104-CM.

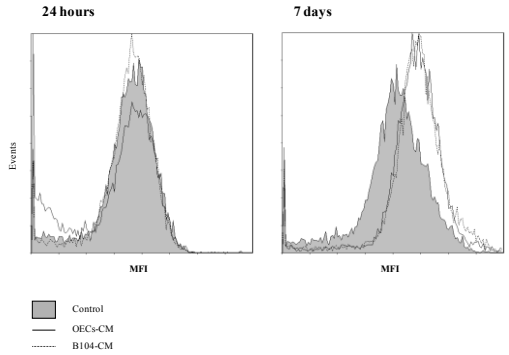

Fig. 45. Nestin (neural stem cell marker) expression by flow cytometry of control and OECs-CM or B104-CM treated AT-MSCs 24 hours and 7 days after the treatment. A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at λ= 488 nm and fluorescence was monitored at λ= 525 nm. Fluorescence was detected using logarithmic amplification. Mean fluorescence intensity (MFI) values were calculated and recorded automatically.

- PGP 9.5

Percentage of positive cells for PGP 9.5 was invariably high (above 97%) at any time and in all samples, either in controls or in conditioned media. MFI at 24 hours was quite strong, particularly in OECs-CM and B104-CM cultures. At 7 days, it increased in B104-CM and markedly in OECs-CM cells, whereas in controls it showed values similar to 24 hours.

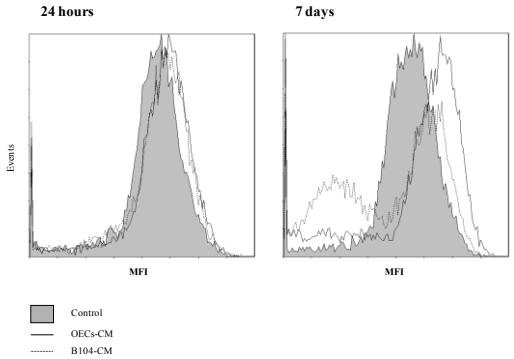

Fig. 46. PGP 9.5(neural stem cell marker) expression by flow cytometry of control and OECs-CM or B104-CM treated AT-MSCs 24 hours and 7 days after the treatment. A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at λ= 488 nm and fluorescence was monitored at λ= 525 nm. Fluorescence was detected using logarithmic amplification. Mean fluorescence intensity (MFI) values were calculated and recorded automatically.

- MAP2

Percentage of positive cells for MAP2 was very high at 24 h (about 95%) in all samples. MFI at 24 hours showed comparable values in all cultures. At 7 days, a marked increase was observed particularly for OECs-CM cultures, but in any case, signal was higher for both conditioned cultures.

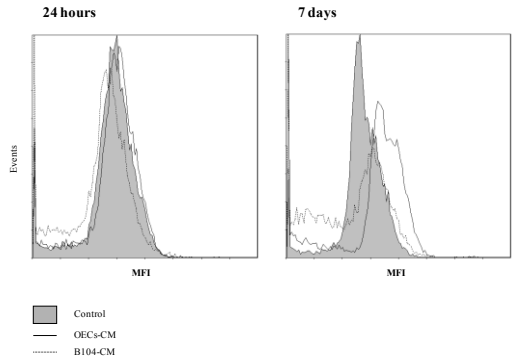

Fig. 47. MAP2 (neural stem cell marker) expression by flow cytometry of control and OECs-CM or B104-CM treated AT-MSCs 24 hours and 7 days after the treatment. A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at λ= 488 nm and fluorescence was monitored at λ= 525 nm. Fluorescence was detected using logarithmic amplification. Mean fluorescence intensity (MFI) values were calculated and recorded automatically

- GFAP

Percentage of positive cells for GFAP was high at 24 hours (about 65%) in control cultures and in cells treated with B104-CM, but was greater for OECs-CM cultures (about 88%). At 7 days percentages were low in OECs-CM and B104-CM. MFI at 24 hours were similar in controls and in B104-CM cultures, whereas it was higher in OECs-CM cultures. At 7 days a progressive decrease was detected for all samples considered.

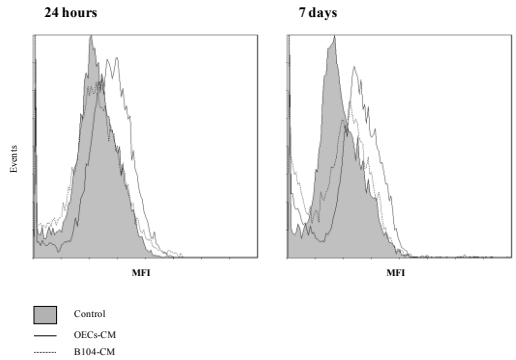

Fig. 48. GFAP (astrocyte differentiation marker) expression by flow cytometry of control and OECs-CM or B104-CM treated AT-MSCs 24 hours and 7 days after the treatment. A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at λ= 488 nm and fluorescence was monitored at λ= 525 nm. Fluorescence was detected using logarithmic amplification. Mean fluorescence intensity (MFI) values were calculated and recorded automatically

- A2B5

Percentage of positive cells for A2B5 was low at 24 hours (about 6%) in all cultures. At 7 days percentage was higher in controls, but showed a very marked increase in B104-CM cultures (about 60%) and, especially, in OECs-CM cultures (72%). MFI at 24 hours showed comparable values in all cultures considered, with a slightly higher value for B104-CM cultures. At 7 days, no significant differences were detected for all samples.

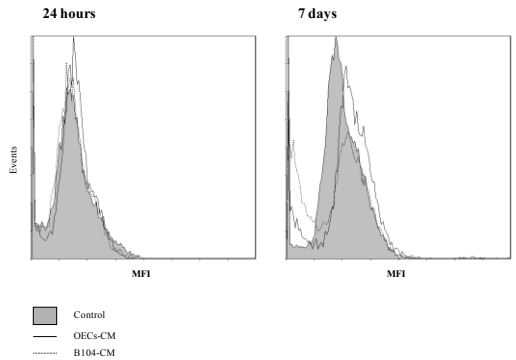

Fig. 49. A2B5 (oligodendrocyte differentiation marker) expression by flow cytometry of control and OECs-CM or B104-CM treated AT-MSCs 24 hours and 7 days after the treatment. A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at λ= 488 nm and fluorescence was monitored at λ= 525 nm. Fluorescence was detected using logarithmic amplification. Mean fluorescence intensity (MFI) values were calculated and recorded automatically.

AT-MSCs can be influenced by the environment versus a neural phenotype.

DISCUSSION

"Credo di poter affermare che nella
ricerca scientifica
né il grado di intelligenza né la capacità di eseguire
e portare a termine il compito intrapreso
siano fattori essenziali
per la riuscita e per la soddisfazione personale.
Nell'uno e nell'altro contano maggiormente la
totale dedizione e il chiudere gli occhi
davanti alle difficoltà:
in tal modo possiamo affrontare
i problemi che altri,
più critici e più acuti,
non affronterebbero."[3]

Rita Levi Montalcini

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3 Cited from Patrizia de Mennato, in La ricerca «partigiana», Libreria CUEM, Milano, 1994.

DISCUSSION

Krabbe disease symptoms appearance and pathology progression are characterized in humans and in animal models: motor defects, cognitive deficits, seizures and early death are well described and attributed to oligodendrocytes loss and demyelination. Key molecule of these phenomena is psychosine, accumulating preferentially in oligodendrocytes as consequence of lysosomal enzyme galactosylceramidase deficiency (Wenger et al., 2001). Much effort has been expended searching for the cellular pathway or target receptors responsible for psychosine effects, but the mechanism has yet to be elucidated.
In this study, we have analyzed different aspects of psychosine interferences within cellular systems, focusing on cellular growth, apoptosis induction and oligodendrocytes differentiation. We used stabilized of oligodendrocytes precursors cell lines and fibroblasts with and without galactosylceramidase mutation and we have shown that:

- psychosine effects is not linked to cellular genome (WT or KD), but they are strongly dependent to concentration and cell sensitivity;
- psychosine treatment causes stop in cellular growth, with an apoptosis induction in cells as observed with analysis of apoptotic index, DNA fragmentation and caspase-3 activation;
- exogenous psychosine interferes in cell homeostasis thought deprivation of survival signalling that interact on cellular membrane, PI3K and PTEN
- the kinase reduction inhibits NF-κB activation, meantime enhance p53 and Bad levels: the activation of the intrinsic apoptotic program is determined;
- psychosine does not active extrinsic apoptosis, because no active-caspase-8 is found;
- psychosine-inducted Cx43 levels justifies rate of apoptosis and the differences in rate of cell responses;
- Cx43 is expressed in cytoplasm of oligodendrocytes precursor cells and is responsible of the undifferentiating phenotype of OPC-TWI;
- AT-MSCs treated with B104- or OECs-CM differentiated to neural fate express markers of progenitor oligodendrocytes.

The present study was undertaken to give a contribution in understanding the molecular events involved in the death of oligodendrocyte by psychosine. Considering that in the infantile form, as in the *twitcher* mouse, symptoms appears quickly, we formed experiments in obtained $A2B5^+$ stabilized cell lines, which are the earlier stage of oligodendrocytes maturation. Furthermore, primary fibroblasts were used as control cells. Firstly, Psychosine treatment was found to induce a dose-dependently arrest of cell growth and proliferation. Effects were not linked to genomic asset or to cellular type, even if different cell sensitivity was observed: high psychosine concentration (100 μM) had a levelling effect on proliferation, while the

lower doses showed a more and more major resistance to toxic effect of FIBRO-WT and –TWI respects to OPCs, as evidenced by various criteria used to measure cell proliferation and induction of death. These findings are in agreement with previously published reports (Tohyama et al. 2001; Jatana et al. 2002). Tohyama et al. (2001) showed that psychosine was cytotoxic to cultured fibroblasts and glia-derived MOCH-1 cells and that the mode of cell death was apoptotic. Appling to our cell models 50 μM of psychosine DNA fragmentation, rounding and cytoplasmic shrinkage and a significant cell detachment, the hallmarks of apoptosis, was observed not only in OPC-TWI, but also in OPC-WT, while rounding cells and a minor intensity of cell detachment were found in FIBROs, showing that psychosine, for its own chemical nature, is capable of interfering in cell membrane conformation. In addition, OPCs stopped growing and were not able to re-establish their proliferative activity after insult removal, whereas a growth-delay of survivor FIBROs seemed to be the consequence of senescent phenotype stimulation. These studies of dose – responses are in agreement with the "psychosine hypothesis", in both OPCs psychosine is fully effective in killing cells at an average of 40 μM corresponding to 40 nmoles/ml. Considering that psychosine concentration in normal brains was 2-3 nmoles/ gram and it is increased up to 10-20 fold in patient brains and in twitcher mouse, the scheme of dose-response underlined in OPC could perfectly reflect the concentration in brain oligodendrocytes. However, morphological analysis indicated the activation of apoptosis in fibroblast, also.

Caspase-3 is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the cascade of events associated with apoptosis. Activated caspase-3 is responsible for the breakdown of several cellular components related to DNA-repair and regulation, such as poly ADP-ribose polymerase (PARP) (Schlegel et al. 1996), and nulear laminins (Lazebnik et al. 1995). Thus, the programmed cell death was confirmed by detecting the active form of caspase-3, as also reported by Tohyama in cultured fibroblasts and glia-derived MOCH-1 cells (Tohyama et al., 2001), while low levels of apoptosis were detected in murine T-cell lymphoma lines and in cell lines derived from human childhood T-cell acute lymphocytic leukaemia exposed to psychosine (Malone et al., 2004); similar conditions were reported for peripheral blood mononuclear cells from KD patient (Formichi et al., 2007), suggesting that its toxic effects are probably restricted to myelinating cells. Indeed in this work, our data confirmed that the apoptosis psychosine-activated was not cell-specific and not GALC mutation-dependent, because high concentration of lipid determined caspase activation in all models, whereas a relationship between concentration and damage induction was established. This could reflect the situation in *twicher* mouse and Krabbe patient: psychosine exerts its toxicity in cells where it is accumulates and the intensity of damage directly refers to cell sensitivity. In order to define the psychosine-inducted toxicity we examined different pathway, making a particular attention to all molecular mechanism that are linked to membrane stability. We have shown psychosine affected cell survival thought deprivation of PI3K-mediated survival signalling that interact on cellular membrane and orchestrate the

apoptotic cascade factors. Generally, PI3K pathway plays a central role in cell, as suggested by the observation that PI3K inhibitors are known for survival block. PI3K enzyme is normally present in cytosol and can be activated directly by an Trk receptor, or indirectly through activated Ras. In the active form, PI3K catalyses the formation of the lipid 3'-phosphorylated phosphoinositides, which regulate localization and activity of Ser/Thr kinase Akt by phosphorilation (Philpot et al, 1997). PI3K activity orchestrates a series of phosphorilation that are necessary for oligodendrocyte cell survival (Cui et al., 2005; Ebner et al., 2000; Ness and Wood, 2002; Vemuri and McMorris, 1996). Among molecules PI3K-activating, there is IGF-1 that is involved in oligodendrocytes maturation (Palacios et al., 2006). In twitcher mouse was reported that psychosine accumulates in bones, where either maybe by itself as an endogenous factor or in conjunction with a decrease of plasma IGF-1 levels, preferentially impaired osteoclastic function, and hence stunted bone growth (Contreras et al., 2010). IGF-1 provides protection against psychosine-induced apoptosis in cultured mouse oligodendrocyte progenitor cells using primarily the PI3K/Akt pathway (Zaka et al., 2005). It is known that PI3K regulation is finely mediated by Phosphatase and Tensin homolog (PTEN). This protein acts as a tumour suppressor by eliciting apoptosis and G1 cell cycle arrest (Weng et al., 2001a). It is a dual-specificity phosphatase that dephosphorylates both lipid and protein substrates: it is able to dephosphorylate the PI3K product (Cantley and Neel, 1999), the growth factor-induced Shc phosphorylation and it suppresses the MAP kinase signalling pathway, also (Weng et al, 2001b). Oligodendrocyte PTEN is required for myelin and axonal integrity, not for remyelination (Harrington et al., 2010) and suppression of PTEN expression may provide neuroprotection in CNS after certain injuries (Gary et al., 2002; Lee et al, 2004; Ning et al, 2004).

Our results directly showed a PI3K activity reduced, extremely linked to PTEN up-expression in all cell models, except in 20 μM psychosine-treated FIBRO-WT. In this case in the balance PI3K/PTEN, the survival factor had a major activity, but higher concentration eliminated the residual PI3K activity. These data confirmed a predominant psychosine role in altering the integration of signals for promotion and stop growing, during the process of intracellular accumulation. This data could be useful for justifying the difference in caspase-3 activation: as modulator of cell survival PI3K takes part to apoptosis symphony. In fact, PI3K activity negatively regulates the proapoptotic Bad and p53, and on the other hand stimulates the NF-κB survival pathway by phosphorylation of IκB (Gewies, 2003).

Bad is a pro-apoptotic member of the Bcl-2 family, PI3K-mediated phosphorilation of Bad promotes its interaction with the chaperone protein 14-3-3,which sequesters Bad in the cytoplasm and inhibits proapoptotic activity (Datta et al, 1997), while the unphosphorylated form can bind to Bcl-xL and thus block cell survival (Yang et al, 1995). p53 is a pro-apoptotic transcription factor inactivated by PI3K/Akt phosphorylation of the oncogene Mdm2 which thereby is activated for inhibition of p53 (Mayo and Donner, 2002), In addiction, the PI3K activity is also

required for stimulating the NF-κB survival pathway by phosphorylation of its inhibitor IκB kinase α (IKK α). Both this events are often inappropriately activated or overexpressed in the process of cancer transformation (Chene, 2003; Orlowski and Baldwin, 2002). The treatment with psychosine dramatically reduced PI3K activity in OPCs and FIBRO-TWI at all concentration, determined a great amount of unphosphorilated free Bad, an high level of p53 and a down-expression of NF-κB free form. All this hallmarks are finely associated to activation of caspase-3. In FIBRO-WT the remaining PI3K seemed to be addressed in blocking Bad, while p53 and NF-kB levels were altered.

As regard the psychosine action on NF-κB there are some aspect to underline. The role of NF-κB in mediating cell death is controversial: authors have demonstrated psychosine potentiates cytokine-mediated induction of iNOS by involving various transcription factors, without attenuating the activation of NF-κB (Giri et al., 2002). Other researcher found psychosine down-regulates the LPS-induced trans-activation of NF-κB, thereby inhibiting this antiapoptotic pathway (Haq et al., 2003). Also, psychosine alone is described capable to decrease the NF-κB nuclear translocation when compared with control and it was further corroborated by the finding that LPS-mediated IκBa degradation was attenuated by the psychosine treatment and that LPS-mediated translocation of NF-κB subunits, p65 and p50 was inhibited in the presence of psychosine (Haq et al., 2003). These findings contribute to our speculation that psychosine-modified pathways are directly dependent to concentration and cell sensitivity at cytoplasmatic membrane levels.

Considering that psychosine firstly damages cell membrane, we analyzed connexin 43 expression profile in our cell models, because Cx43 can be localized both in cell membrane and in cytoplasm and there is evidence that Cx43 GJ channels, hemichannels and protein *per se* are involved in many cellular processes such as homeostasis, metabolic support, electrical coupling, and enhancement of tissue response, control of cell proliferation, differentiation, survival and apoptosis (Kumar and Gilula, 1996; Carette et al, 2013). Still today, no study was performed for evaluating Cx43 in oligodendrocytes precursors, while different investigations were made in astrocytes, in which Cx43 is an element of both GJ structure (Zhang et al., 2013) and hemichannel (Wang et al., 2013). Cx32 is described as oligodendrocytes GJ protein (Nagy and Rash, 2000). Our intent in Cx43 investigation followed a deep study of known connexins cellular expression in brain (Condorelli et al., 2003). Authors demonstrated that only Cx45 and Cx36 mRNAs are localized in neuronal cells, and it was found Cx32 and Cx43 were up-regulated in non-neuronal cells in the same brain region involved in kainate seizure-inducted apoptotic lesion. Moreover, authors revealed Cx43 was widely expressed in all the grey and white matter regions, not in neural cell ($NeuN^+$) of adult brain (Condorelli et al., 2003). Thus, we analyzed Cx43 expression in our cellular models with and without toxic psychosine treatment. Both OPCs and FIBROs expressed Cx43. Comparing the GALC-mutated with their

own wild type control, it was found that OPC-TWI showed a higher not significant basal expression of protein, while FIBRO-TWI a down expression. OPCs showed particular signal intensity in regions corresponding to Golgi apparatus around the nucleus. The psychosine added in culture medium for 24 hours determined an accumulation of Cx43 in intracytoplasmatic and nuclear clusters, without GJ involvement. Like most integral membrane proteins, connexins are synthesized in association with the endoplasmic reticulum and traffic through the Golgi apparatus. However, Cx43 atypically delays oligomerization into a hexameric hemi-channel or "connexon" until reaching the trans-Golgi network (Musil et al., 1993): we can suppose an hemichannel involvement during psychosine treatment without excluding protein action *per se*. The antibody used for Cx43 detection recognizes the C-terminal domain of Cx43 that is cytoplasmatyic and it is thought to play key regulatory roles and provide for sites of protein-protein interaction (Solan and Lampe, 2009). In Cx43, the C-terminal domain appears to be the primary region which can be phosphorylated by PKC in response to src activation (Solan et al., 2008) It has previously been demonstrated that psychosine can inhibit the activity of protein PKC (Hannun and Bell, 1987), and that this inhibition has shown implicated to alter PKC-dependent phosphorylation of myelin basic protein in oligodendrocytes (Vartanian et al., 1989). Moreover, Schwann cells from *twitcher* mouse have impaired PKC activity, which results in suppressed proliferation (Yoshimura et al., 1993, Yamada et al., 1996). Thus, we cannot exclude Cx43 levels in psychosine-treated cells are a result of PKC impaired activity, because still today we have not data about Cx43 phosphorilation state. In any case, levels of this protein, in our conditions, can justify and are linked to the dose-related differences in psychosine-inducted damage. The new and appealing findings were confirmed by brain protein. Respect to wild-type, at symptoms appearance, *twitcher* mice showed a twofold higher expression of Cx43, at proximity of death, they showed significant lower protein levels. This last event could be explained by the oligodendrocytes loss and the activation of a strongly astrocytes-mediated innate immune response to cellular RNA that is released as a result of tissue damage (Zhao et al, 2006). In addition, Zhao and colleagues showed that loss of Cx43 for the innate immune response of astrocytes could be restored with NF-κB and PI3K inhibitors. In our OPCs, both these pathways were blocked by psychosine in a dose-dependent manner, bringing to the activation of apoptosis in cells made more susceptible through Cx43 up-regulation (Fig. 50).

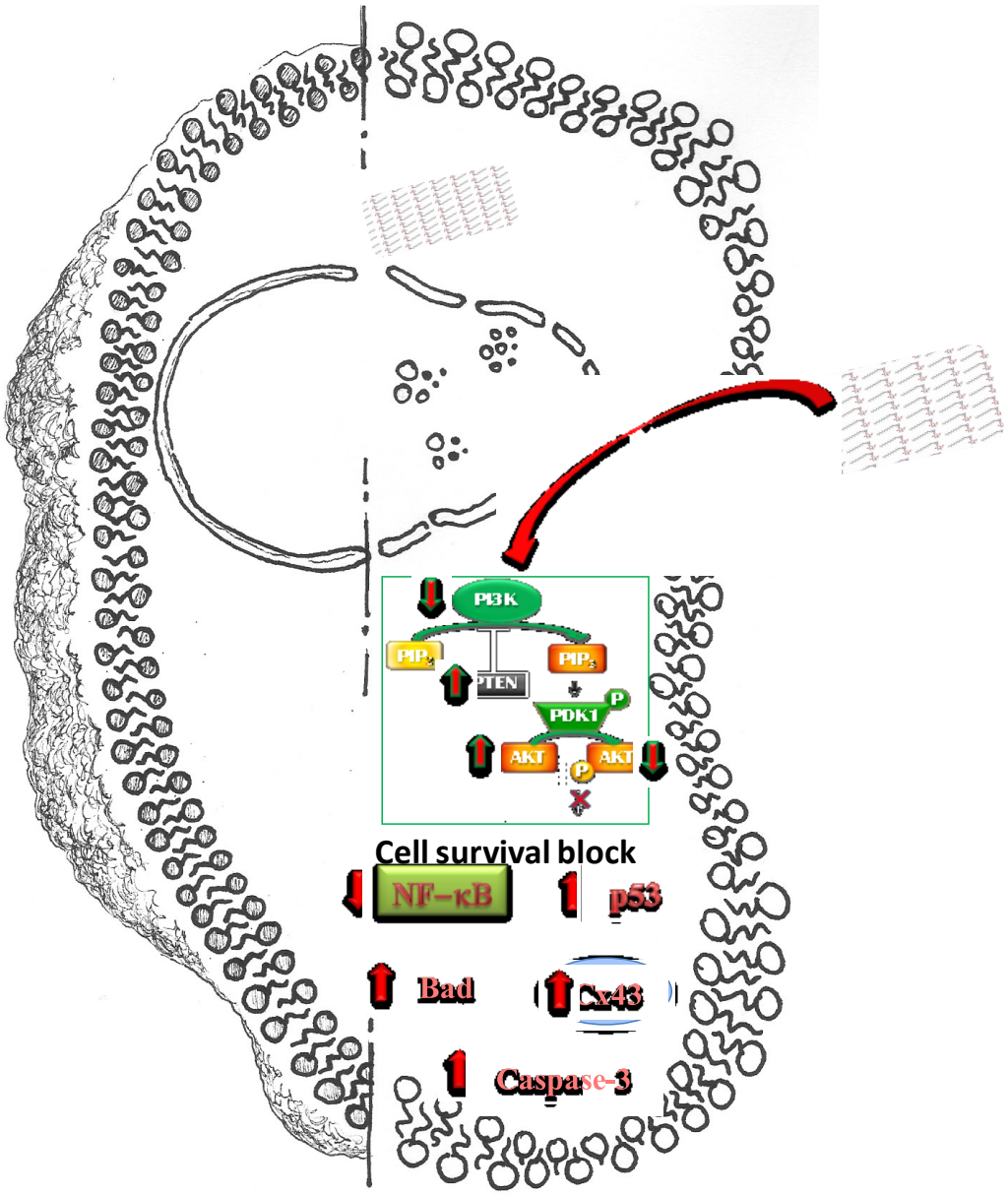

Fig. 50. Molecular mechanism involved in psychosine-inducted toxicity

The analysis of brain development underlined that Cx43 was widespread expressed at P1 and thereafter increased progressively from P1 to P14 restricted to non-neuronal cells. Indeed, specific signals for oligodendrocytic Cx32 mRNA was detected only at P14 prevalently in white matter and never colocalized in NeuN-positive cells (Condorelli et al., 2003). This simple observation validates our findings in the Cx43 modulation role during oligodendrocytes maturation process. For the first time, it was verified that OPC-WT differentiates into a mature phenotype with loss of Connexin43. Under the same condition, GALC-mutated oligodendrocyte precursor do not express marker of maturity, they became multinuclear and Connexin43 levels remain higher than control, suggesting that psychosine is

accumulating in early steps of oligodendrocyte precursor cell maturation machinery (Fig. 51).

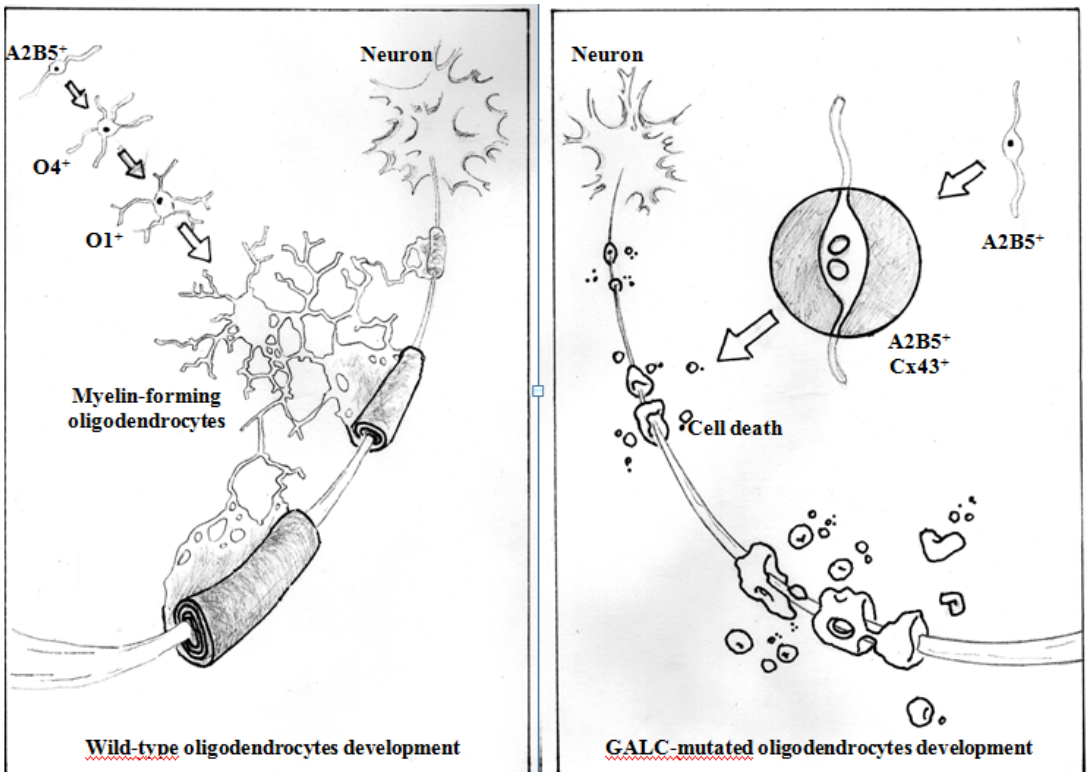

Fig. 51. Cx43 involvement during OPCs maturation

Our results fit well with the recent demonstration that accumulation of endogenous psychosine under GALC deficient Krabbe conditions impedes oligodendrocytes differentiation process both by decreasing the expression of myelin lipids and protein and by inducing the cell death of maturating cell (Won et al., 2013).

Taken together, this finding conducted the rational necessity to find cell that may substitute these ones loss in Krabbe disease. Recent advances in human stem cell biology and the optimization of protocols for in vitro differentiation of stem cells into different cell lineages have opened new possibilities. Mesenchymal stem cells isolated from adipose tissue (AT-MSCs) were treated with B104- or OECs-conditioned medium (CM). The results confirmed that it is possible to isolate clonogenic adult stem cells expressing mesenchymal and pluripotent state associated markers from adipose tissue.Moreover, AT-MSCs treated with B104- or OECs-CM differentiated to neuronal fate, expressed markers of progenitor and mature neurons (nestin, PGP 9.5 and MAP2) and displayed morphological features resembling neuronal cells. Nestin has traditionally been noted for its importance as a neural stem cell marker. It is required for brain development and for survival, renewal and mitogen-stimulated proliferation of neural progenitor cells. It promotes the disassembly of phosphorylated vimentin intermediate filaments (IF) during mitosis and plays a role in the trafficking and distribution of IF proteins and other cellular factors to daughter cells during progenitor cell division. Therefore, nestin is

expressed both in embryonic and adult CNS stem cells. Nestin expression in the embryonic CNS is spatially and temporally linked closely to the proliferating cell state, which makes it a commonly used marker for this cell type. Similarly, nestin expression is a hallmark of adult CNS stem cells, both in vivo and when the adult CNS stem cells are cultured in vitro under non differentiating conditions. Nestin expression is downregulated when CNS stem cells differentiate and other members of the intermediate filament gene family are expressed, primarily neurofilament in neurons and glial fibrillary acidic protein (GFAP) in astrocytes. The down-regulation of nestin protein and its mRNA correlates with multipotential cells leaving the proliferative state and differentiating into astrocytes, which express glial fibrillary acidic protein (GFAP), or neurons, which express neurofilament proteins (Dahlstrand et al., 1995; Xu et al., 2008).

In the present study, the expression of the neural stem cell marker nestin was detected in AT-MSCs treated with B104- or OECs-CM. Furthermore, immunofluorescent and cytofluorimetric analysis revealed that the treated AT-MSCs had the ability to differentiate into neurons. The expression of the mature neuronal markers PGP 9.5 and MAP2, even if in presence of expression of nestin probably due at the early time of differentiation (24 hours and 7 days), indicated that the AT-MSCs had been differentiated into more mature neuronal cells. PGP 9.5 (also known as UCHL1), a 27 kDa ubiquitin-protein hydrolase, is expressed in the cytoplasm of neurons and cells of the diffuse neuroendocrine system. PGP 9.5 hydrolyses a peptide bond at the C-terminal glycine of the proteolysis tagging protein ubiquitin, thereby generating/recycling ubiquitin monomers, essential in the ubiquitin-proteasome cytoplasmic protein degradation system (Day a and Thompson, 2010). Microtubule-associated protein 2 (MAP2) is a major component of the neuronal cytoskeleton and is known to promote the assembly and stabilization of microtubules, functions which have important implications in neuronal differentiation (Przyborski and Cambray-Deakin, 1995). MAP2 antibody (SMI 52) reacts with microtubule-associated protein 2 (MAP2). SMI 52 recognizes neuronal cell bodies and dendrites in tissue sections and cell cultures. Moreover, the results demonstrated that AT-MSCs treated with conditioned media result to be lower positive for GFAP and A2B5. GFAP, a astrocyte marker and a class-III intermediate filament, is a cell-specific marker that, during the development of the central nervous system, distinguishes astrocytes from other glial cells. A2B5 is a cell surface ganglioside epitope expressed in developing thymic epithelial cells, oligodendrocyte progenitors and neuroendocrine cells. This study demonstrated that AT-MSCs can be influenced by the environment, indicating that these cells can respond to environmental cues also versus a neuronal phenotype. As potential material for cell replacement therapy in central nervous system degenerative diseases, this culture system may therefore offer many advantages, including the ability to expand, safety test, and bank these cells before transplantation, but they ability *in vivo* cannot be predicted because the milieu could conditioner their phenotype.

CONCLUSIONS AND FUTURE PERSPECTIVES

This study was originally designed to determine molecular effects of psychosine in different cellular systems, especially in oligodendrocytes precursors. It was demonstrated that in Krabbe disease, psychosine affects negatively the cells membrane leading to loss of viability and start of programmed cell death. For the first time, it was established that oligodendrocyte precursor cells express Connexin43. This advance provides new information on unexplored molecular mechanisms of Krabbe disease hallmarks. The levels of Connexin43 can justify the dose-related differences in psychosine-induced damage and the delay in oligodendrocytes maturation program. Although more research is required to fully elucidate the Connexin43 up-regulation and its intracellular trafficking in maturating oligodendrocytes, this research provides new information on the use of Connexin43 as a new potential therapeutic target in Krabbe disease. Finally, the possibility of AT-MSCs application in "cell replacement therapy" might be investigated *in vivo*, using *twitcher* mouse.

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