Review

Glial cells involvement in spinal muscular atrophy: Could SMA be a neuroinflammatory disease?

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ABSTRACT

Spinal muscular atrophy (SMA) is a severe, inherited disease characterized by the progressive degeneration and death of motor neurons of the anterior horns of the spinal cord, which results in muscular atrophy and weakness of variable severity. Its early-onset form is invariably fatal in early childhood, while milder forms lead to permanent disability, physical deformities and respiratory complications. Recently, two novel revolutionary therapies, antisense oligonucleotides and gene therapy, have been approved, and might prove successful in making long-term survival of these patients likely. In this perspective, a deep understanding of the pathogenic mechanisms and of their impact on the interactions between motor neurons and other cell types within the central nervous system (CNS) is crucial. Studies using SMA animal and cellular models have taught us that the survival and functionality of motor neurons is highly dependent on a whole range of other cell types, namely glial cells, which are responsible for a variety of different functions, such as neuronal trophic support, synaptic remodeling, and immune surveillance. Thus, it emerges that SMA is likely a non-cell autonomous, multifactorial disease in which the interaction of different cell types and disease mechanisms leads to motor neurons failure and loss. This review will introduce the different glial cell types in the CNS and provide an overview of the role of glial cells in motor neuron degeneration in SMA. Furthermore, we will discuss the relevance of these findings so far and the potential impact on the success of available therapies and on the development of novel ones.

1. Introduction

Spinal Muscular Atrophy (SMA), also termed 5q-SMA to distinguish from less common forms, is a hereditary neuromuscular disease, caused by loss-of-function mutations in the gene Survival Motor Neuron 1 (*SMN1*), causing the reduction of SMN protein, and characterized by hypotonia and weakness, which might prove fatal in early childhood (Faravelli et al., 2015; Pearn, 1978; Sumner et al., 2016). SMA has been considered for a long time an incurable deadly disease, until recent therapeutic advances, namely antisense oligonucleotides and gene therapy, have been successful in modifying its natural history from a rapidly fatal disease to a condition in which long-term survival is likely (Parente and Corti, 2018). At a pathological level, SMA is defined by the degeneration of anterior horn cells in the spinal cord and motor nuclei in the lower brainstem, which results in a progressive, diffuse and symmetric muscle weakness and atrophy (Pearn, 1978). However, despite the traditional belief that SMA is a motoneuronal-restricted disease, recent evidence advocates the involvement of many different cells and systems alongside with motor neurons in the pathogenesis of this disease. Glial cells are the most abundant cell type in the central nervous system (CNS), surrounding neurons and providing nutritional and trophic support for them (Papadimitriou et al., 2010). These cells also play an important role in neuronal communication and neuroinflammation (Papadimitriou et al., 2010). Although the presence of cell-autonomous motoneuronal toxicity in this disease has been established, growing evidence supports the possibility that, in addition to that, glial dysfunction and glial-mediated inflammation might be able to compromise neuronal survival and promote progression and propagation of the degenerative process (Philips and Rothstein, 2014). Arguably, it can be hypothesized that, under specific circumstances, neuroinflammatory cells - astrocytes and microglia – could directly start the process of neurodegeneration. In this review, we will briefly recapitulate the different glial cell types and their role within the CNS, and we will provide an overview of the different mechanisms implicated in glial-mediated

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neuronal damage. The understanding of these pathways might pave the way to the development of therapies specifically targeting glial dysfunction in SMA.

2. Spinal muscular atrophy

2.1. Clinical and genetic features of 5q-Spinal Muscular Atrophy

Notably, 5q-SMA is the most common cause of child and infant mortality with a neuromuscular etiology (Brzustowicz et al., 1990; D'Amico et al., 2011; Pearn, 1978) It is characterized by the selective loss of spinal cord motor neurons (and sometimes also brainstem motor nuclei), muscular atrophy and muscular failure (D'Amico et al., 2011).

In the majority of cases, the genetic culprits are biallelic deletions or mutations in the Survival Motor Neuron 1 gene (SMN1) on chromosome 5 (5q13.2), leading to deficiency of the SMN protein (Frugier et al., 2002). Among them, the most common mutation (present in almost 95% of the patient with SMA) is the deletion of exon 7 in SMN1 (Frugier et al., 2002). Although its biological roles have not been completely elucidated yet, it is known that SMN, in collaboration with partner proteins, catalyzes the assembly of snRNPs (which are building blocks for pre-mRNA splicing) (Juntas Morales et al., 2017). Moreover, different studies suggest that SMN could have also a role in axonal transport and in the formation, maintenance and remodeling of the neuromuscular junction (NMJ) (Juntas Morales et al., 2017).

Differences in SMN protein functioning and in disease phenotypes seem to be related, at least in part, to the residual activity of SMN protein, which in turn relates to the number of copies of a similar gene called *SMN2* (Frugier et al., 2002). This gene differs from *SMN1* for 1% of the sequence, presenting a thiamine in place of a cytosine at codon 280 in exon 7 (Lorson et al., 1999). This modification determines the production of a truncated, non-functional SMN protein from 85 to 90% of the *SMN2*-derived mRNA. The remaining 10–15% of *SMN2*-derived mRNA can instead produce a full-length and functioning SMN protein, thus generating a very small amount of functional SMN protein (Lorson et al., 1999).

For this reason, the loss of function of *SMN1* can be partially compensated by the *SMN2*-derived protein synthesis. The number of *SMN2* copies is not constant and varies between different individuals, thus the variation in *SMN2* copy number partially explains the phenotypic variability within SMA patients (D'Amico et al., 2011; Feldkötter et al., 2002). Indeed, disease severity is usually inversely proportional to *SMN2* gene copy number. Milder phenotypes are associated with the presence of 3 or more copies of *SMN2*. The expression of 8–16 *SMN2* copies, that has been tested in a murine model, completely eliminated the pathologic phenotype in mice (Monani et al., 2000).

While this is the genetic mechanism underlying the most common SMA form, there are also different forms of disease lacking *SMN1* mutation, which present marked clinical and genetic heterogeneity. Non-5q SMA subtypes are thoroughly reviewed in (Peeters et al., 2014). Nonetheless, this review will focus on 5q-SMA, as it is the most common and widely studied form and the target of the most recent therapeutic innovations.

5q-SMA is classified in 5 types: 0, 1, 2, 3, and 4, depending on the age of onset (prenatal, infantile, adult) and on clinical features (most severe, severe, intermediate, mild). Common clinical features are symmetric, and predominantly proximal weakness, especially in the lower limbs, and absent or markedly reduced deep tendon reflexes (DTR) (D'Amico et al., 2011). In some cases (especially in types 0 and 1) SMA is correlated with progressive respiratory failure and sleep respiratory disturbances requiring non-invasive ventilation (NIV) (Boentert et al., 2017; Grychtol et al., 2018).

As mentioned above, SMA is clinically classified in types depending on age of onset and disease course. SMA type 0 and type 1 patients can display several systemic features, such as autonomic dysfunction, cardiac failure and cutaneous necrosis (Simone et al., 2016). These characteristics are even more evident in transgenic mouse models (Shababi et al., 2010).

The frequency and severity of systemic involvement increases proportionally to the reduction of SMN protein levels. This relation is possibly due to the role of SMN in the RNA metabolism pathways.

Multi-organ involvement in SMA include a variety of symptoms, ranging from vascular and cardiac alterations, to immune system abnormalities and peripheral nerve involvement (Simone et al., 2016). Additionally, severe SMA patients may present several cardiac alterations caused mainly by congenital anomalies that emerge early in cardiogenesis. Thus, a current suspect is that SMN may play a key role in cardiogenesis. Other symptoms that can be found in patients with SMA are hypocalcemia-related osteopenia, pancreatic defects, alterations in the fatty acids metabolism and hepatic impairment, and reproductive system defects (Abati and Corti, 2018). Systemic symptoms in SMA patients have been thoroughly reviewed by Simone and colleagues in Simone et al. (2016).

In the last years, we witnessed a true revolution in the field of SMA therapeutics, and of motor neuron diseases in general. In 2016, an antisense oligonucleotide called Nusinersen was approved by the Food and Drug Administration (FDA) and, subsequently, by the European Medical Agency (EMA) for the treatment of SMA (US Food and Drug Administration, 2016). Nusinersen works by binding to an intronic sequence in exon 7 in *SMN2* mRNA, thereby determining skipping of exon 7 and production of a full-length, functional protein (Finkel et al., 2017). Furthermore, in 2019 the FDA approved a second drug, *onasemnogene abeparvovec*, an adeno-associated virus (AAV)-based gene therapy that delivers a functional SMN full-length cDNA to motor neurons, for children less than 2 years (Hoy, 2019). Thus, a deep understanding of the developmental effects of SMN deficiency in all cell types, is necessary in order to fully monitor treated patients and to identify the correct timing and delivery methods for these therapies.

2.2. Is SMA a multisystemic disease?

As mentioned above, SMA is a neurodegenerative disease involving motor neurons, but many studies suggest that different organs take part in its phenotype, especially in the most severe forms, in which the depletion of SMN protein is profound.

The contribute of non-motor neuronal cells to the pathogenesis has been demonstrated by the fact that SMN restoration in motor neurons in *in vivo* models is not sufficient to completely restore muscle functionality. Although the restoration of *SMN1* in SMA mice under the motoneuronal-specific choline acetyltransferase (ChaT) or homebox 9 (HB9) gene proved able to revert synaptic degeneration and reduce muscle atrophy (Gogliotti et al., 2012; Lee et al., 2012a; Martinez et al., 2012), while muscle-restricted expression could not (Martinez et al., 2012), only minimal improvements in motor performance and survival were observed. The limited efficacy of neuronal-only, *SMN1*-enriched transgene models could be ascribed to failure of these models to address disease mechanisms other than neurodegeneration, such as autonomic dysfunction that was a prevalent morbidity cause in these mice (Gogliotti et al., 2012). Additional evidence of the benefit of SMN1 restoration not restricted to motor neurons comes from the observation that expression of SMN under the prion promoter (PrP), which is highly expressed both in neurons and in astrocytes, provokes a dramatic improvement in animal survival (average 210 days) and a normal lumbar motor neurons count (Gavrilina et al., 2008). These and other data suggest that not only motoneurons, but also astrocytes, sensory neurons, Schwann cells and myocytes can contribute to the expression of the disease and the loss of MNs (Jablonka et al., 2006; Mentis et al., 2011).

In addition to that, Hua and collaborators demonstrated that systemic administration of oligonucleotides that correct *SMN2* splicing almost completely rescued disease signs, in particular peripheral tissues necrosis, in mild SMA mice, and significantly extended survival of

severe SMA mice, with noticeable improvements in motor neuron survival, neuromuscular junction integrity, and motor function; this effects were not achieved with intracerebroventricular (ICV) administration (Hua et al., 2011). Accordingly, they conclude that the SMA phenotype in murine models is not the result of a selective motor neuron defect alone.

2.3. Involvement of non-motor neuronal neural cells in central and peripheral nervous system

As we mentioned, several lines of evidence seem to suggest that non only motor neurons, but also other cell types may be affected by *SMN1* depletion. Notably, in milder SMA forms with higher levels of SMN non-motor neuronal and systemic symptoms are less evident, while severe forms show a higher percentage of these complications (Shababi et al., 2010; Simone et al., 2016). Therefore, it could be hypothesized that there is a variable susceptibility threshold for SMN reduction in different types of cells (Simone et al., 2016).

For instance, early dysfunction of sensory neurons and disruption of sensory-motor circuits has been widely demonstrated in preclinical studies (Imlach et al., 2012; Jablonka et al., 2006; Lotti et al., 2012; Mentis et al., 2011). Indeed, abnormal conduction velocity and sensory neuronopathy with neurogenic changes at muscle biopsy are very common in SMA-1 patients (Anagnostou et al., 2005; Omran et al., 1998; Rudnik-Schöneborn et al., 2003). A recent study has revealed that motor neuron degeneration follows a dysregulation of mRNAs which are necessary for the formation of synaptic connections between sensory and motor circuitries in SMA mice spinal cord (Zhang et al., 2013). Indeed, deep RNA sequencing on human SMA motor neurons derived from patients human induced pluripotent stem cells (iPSC) revealed specific altered gene splicing/expression. Many deregulated genes, such as the neurexin and synaptotagmin families, are implicated in critical motor neuron functions (Rizzo et al., 2019)

Moreover, it was shown that the first symptomatic stages in SMA are characterized by morphological and functional abnormalities of neuromuscular synapses (Dachs et al., 2013; Kariya et al., 2008; Kong et al., 2009; Lee et al., 2011).

In addition to that, patients with SMA suffer from esophageal reflux, constipation and delayed gastric emptying, which might be a direct consequence of SMN deficiency in neurons of the enteric nervous system (ENS), rather than a secondary event. In murine models, SMN deficiency in the ENS resulted in constipation, delayed gastric emptying, slowing of intestinal transit and reduced motility of the colon (Gombash et al., 2015). In particular, SMN deficiency causes interruption of ENS signaling to colon smooth muscle. Defects in cardiac autonomic innervation, with reduced sympathetic neuron staining and axon branches, were also observed in SMA mice (Gogliotti et al., 2012; Heier et al., 2010).

3. Motor neurons involvement in SMA

Traditionally, the main anatomopathological manifestation of SMA was the motor neurons degeneration within the anterior horn of the spinal cord. The same degenerated neurons, rich in phosphorylated neurofilaments, have also been found at the level of the thalamus, of the dorsal root ganglia and of the motor nuclei of the extrinsic ocular musculature (D'Amico et al., 2011).

Although SMN protein influences the RNA processing functions in all cells, it explicates its prominent role and pathogenic effect in motoneurons through mechanisms that are still not completely understood.

As mentioned above, SMN contributes to the formation of snRNPs, and thus loss of SMN protein determines aberrant splicing and widespread transcriptional changes to which motor neurons are especially vulnerable (Bäumer et al., 2009; Corti et al., 2012; Ng et al., 2015; Rizzo et al., 2019). Furthermore, SMN regulates mRNA translation and trafficking of RNA-binding proteins in neurites as well, thus impairing normal neurite function and extension in *in vitro* SMA models (Fallini et al., 2014, 2011; Jablonka et al., 2007; Rossoll et al., 2003) Recently, SMN was shown to be implicated in the biogenesis of microRNAs (miRNAs), which could also explain the numerous pathways that are affected by SMN loss (Magri et al., 2018). Thereby, its deficiency could result in the alteration of the expression of motor neuron-specific selected miRNAs.

In addition to RNA metabolism dysregulation, several studies have shown the presence of an assembling defect of neurofilaments in affected motor neurons, and have advanced the hypothesis that this defect could influence the formation of synapses and disturb the neuron-glia relationship (Chou and Nonaka, 1978). A reduced positive response to synaptophysin in these motor neurons has been reported, confirming the loss of synapses.

Novel murine models expressing a reduction of the SMN protein specifically in the motoneuronal progenitors were developed in order to test the effects of a motoneuronal-specific SMN reduction (Park et al., 2010a). To this aim, Park and colleagues performed a selective depletion of the SMN protein in spinal motor neuron progenitors expressing the transcriptional repressor Olig2 (Olig2-Cre transgenic mice) (Park et al., 2010b). In this study the simple exhaustion of the SMN protein in spinal motor neuron progenitor cells was shown to be sufficient to cause many of the morphological, functional and phenotypic characteristics of human SMA. Progressive silencing caused not only early distal defects of the NMJ, but also impairment of central synapses and degeneration of the motor neurons with consequent muscular atrophy, suggesting that the pathology of the motoneurons in SMA is, at least in part, a cell-autonomous effect of SMN insufficiency. As well as affecting peripheral neuromuscular synapses, the depletion of motoneuronal SMN severely impairs central synaptic integrity.

However, despite the presence of a clear SMA-like pathologic phenotype in these mouse lines, the disease was not as severe as in lines with a ubiquitous SMN depletion. Intriguingly, Olig2-Cre mice did not progress inexorably to death like their SMN-lacking counterparts, but after acquiring motor phenotype they reached a steady state. One potential explanation for this finding is the role of non-nervous CNS cells in sustaining and spreading initial motoneuronal pathology. Supporting this hypothesis, previous data have shown that isolated motor neurons from SMA mice spinal cord do not die when cultured alone, although they die *in vivo* (Rossoll et al., 2003). Moreover, increasing evidence suggests that although the expression of SMN in MNs is essential, its recovery is not sufficient to significantly improve survival in SMA mouse models. When SMN is highly expressed in both astrocytes and MNs, survival is maximized (Lee et al., 2012a; McGivern et al., 2013). These data tell us that astrocytes play an important role in the pathogenesis of SMA, and that they could represent an additional target for therapeutic intervention.

4. Basis of glial cells biology

At the beginning of the XX century, when it was first identified, glia was simply considered as "nerve glue" (Jäkel and Dimou, 2017). In the subsequent decades, however, it became clear that glia is actually much more than that, and that glial cells form a heterogeneous group, with various functions and properties which have not been completely understood yet (Jäkel and Dimou, 2017). As mentioned above, glial cells represent the most abundant cell type within the CNS, forming between 33% and 66% of the total brain mass (Jäkel and Dimou, 2017). Their main functions are to provide structural and trophic support to neurons, and to speed nervous conduction by forming myelin coat around axons. Nonetheless, glial cells exert also a variety of additional, less-known activities, such as antimicrobial filtering and guidance to synapse formation. However, the most complex and elusive of glial-dependent functions is perhaps neuroinflammation. The term "neuroinflammation" encompasses a wide range of molecular and cellular processes,

happening within the CNS, that result in the activation of glial cells and in the infiltration of the nervous tissue by immune cells, and that are present in a variety of pathological conditions, including SMA (Papadimitriou et al., 2010).

Glial population can be divided in 4 groups: microglia, astrocytes, oligodendrocytes and NG2-glia (progenitors of the other subgroups). Astrocytes, oligodendrocytes and NG-2 progenitors originate from ectodermal tissue, whereas microglia derives from the yolk sac (Jäkel and Dimou, 2017). Microglial cells are the main immune-competent cells in the CNS and are deputed to immune surveillance. Microglial cells produce a whole range of pro-inflammatory and anti-inflammatory mediators (cytokines and chemokines) and eliminate not only foreign microorganisms but also compromised cells. After an injury, they are massively activated in the regions of neural loss, determining a condition known as "reactive microgliosis", and working in close interaction with inflammatory T-lymphocytes as well as astrocytes in order to provide inflammatory response (Moisse and Strong, 2006). Microglia can secrete both pro- and anti-inflammatory cytokines and chemokines in different combinations, depending on the received stimuli deriving from surrounding microenvironment and/or from other cells, and thus they can acquire a different phenotype according to these variables ("pro-inflammatory" or M1 *versus* "anti-inflammatory" or M2 phenotype) (Tang and Le, 2016). New evidence suggests that these cells also have an essential role in homeostasis, neuronal survival and synaptic pruning (Cowan and Petri, 2018).

Astrocytes have emerged as critical elements for the maintenance and function of the CNS: they maintain neural functioning, control neural maturation and differentiation and transfer nourishment from the blood stream to neurons through a capillary gap-junctions system (Vasile et al., 2017). Healthy astrocytes greatly participate in the regulation of synaptic function, providing an optimal pre-synaptic environment, triggering synaptic maturation and pruning, maintaining synaptic activity and stability (Pehar et al., 2005; Vasile et al., 2017). For instance, they participate in the glutamate-glutamine circle, taking up glutamate from the synaptic cleft after release from presynaptic terminal, thus preventing excessive postsynaptic stimulation of glutamate receptor which eventually leads to cell death, the so-called "glutamate-mediated excitotoxicity" (Danbolt, 2001). They are also key components of the blood brain barrier (BBB) and may regulate blood flow in the brain trough vasoconstriction and vasodilation (Farina et al., 2007). In addition to that, astrocytes are able to improve neural survival and control neural differentiation and growth by releasing different neurotrophic factors, such as Neurotrofin-3 (NT3) and Brain Derived Neurotrophic Factor (BDNF) (Ojeda et al., 2003; Rudge et al., 1992). Similarly to microglia, astrocytic functions are deeply regulated by immune T-cells, surrounding neurons and possibly oligodendrocytes. After receiving pro-inflammatory stimuli from a nearby biological hazard or from other cells, astrocytes react by increasing the expression of some astrocytic markers, *e.g.* glial fibrillary acidic protein (GFAP) and aldehyde dehydrogenase 1 L1 (ALDH1L1), and by proliferating in a process called astrogliosis (Rothstein et al., 1992; Tawfik et al., 2008).

Oligodendrocytes are glial cells abundantly present within the CNS. Mature oligodendrocytes produce myelin, a specialized structure which encloses axons, thus playing a key role in the efficiency and speed of impulse conduction (Emery, 2010). There is also compelling evidence that oligodendrocytes provide trophic support to neurons, for example through the release of GDNF or by transporter-mediated delivery of metabolic substrates, such as lactate (Lee et al., 2012b; Ubhi et al., 2010). Interestingly, myelination occurs late in neural development and over a prolonged time frame, lasting for the first two decades of life in humans (Mitew et al., 2014). Moreover, it has been shown that myelin remodeling and repair continues throughout life (Young et al., 2013). Oligodendrocytes arise from oligodendrocyte precursor cells (OPCs), which are produced in sequential waves from specific germinal regions. OPCs in turn derive from neural progenitor cells (NPCs) of the germinal layer (Emery, 2010).

At last, the main glial cells of the peripheral nervous system are Schwann cells, which are essential to axon stability and neuronal survival. They release proteins involved in the formation and maintenance of the extracellular matrix (ECM) and form the protective myelin sheath wrapping around axons of peripheral neurons (Chernousov et al., 2008).

5. Role of glial cells in neurodegenerative disorders

In the healthy brain, glial cells remain in a state of quiescence. When the brain is injured, this cells rapidly activate in response to specific molecular signals, developing neuroinflammation (DiSabato et al., 2016).

Neuroinflammation is an innate immune response that occurs within the CNS against harmful stimuli such as pathogens, metabolic toxic waste or chronic stress that occurs in response to trauma, infections and/or neurodegenerative disease (DiSabato et al., 2016). The main cell types contributing to this immune response are microglia and astrocytes. These cells constantly survey the environment through pattern recognition receptors (PRRs). After activation of the PRRs and release of immune molecules, the innate immune system launches inflammatory and regulatory responses in order to counteract infection, injury and maintenance of tissue homeostasis (DiSabato et al., 2016). An example of the beneficial effects of neuroinflammation is that reactive microglia can enhance neuronal repair through "synaptic stripping", a form of controlled-phagocytosis in which it removes pre-synaptic axon terminals from damaged neurons with the aim of directing cell efforts towards repair rather than information processing, both in healthy and diseased brain (Moisse and Strong, 2006). In Amyotrophic Lateral Sclerosis (ALS), this mechanism was shown to occur as an early phenomenon and with ongoing synaptic loss through disease progression, in response to cell injury (Kassa et al., 2018; Zang et al., 2005). In addition, it was shown that, after injection of kainic acid, an excitotoxic damage-inducer, into the striatum of healthy mice, reactive astrocytes could produce high amounts of nerve growth factor (NGF) and lead to the formation of a glial scar, which acts as a physical barrier around the injured area and as a permissive substratum for axonal regeneration (Strauss et al., 1994). Indeed, elevated levels of NGF were observed in brains of patients with Alzheimer's Disease (AD) (Crutcher et al., 1993; Hock et al., 2000). Finally, both microglia and astrocytes play an important role in neuroprotection, as they can preserve neuronal survival through the release of cytokines, neurotrophins and anti-inflammatory growth factors (Zhao et al., 2004). Beneficial effects of stem cell grafts giving rise to growth factor-releasing glial lineages was demonstrated in several ALS mouse models (Abati et al., 2019). Although the evolutionary function of this mechanism is protective, innate immune responses of neuroinflammation can also promote neural damage when excessive or unproportionate. Chronic neuroinflammation is observed at relatively early stages of ALS, AD and other neurodegenerative diseases (Eikelenboom et al., 2002; Heneka et al., 2014; Sargsyan et al., 2005). During chronic activation, the sustained exposure of neurons to pro-inflammatory mediators can cause neuronal dysfunction and contribute to cell death. Activated glial cells can produce toxic molecules (such as ROS and NO) which mediate a noxious action on neighboring neurons (MacMicking et al., 1997).

Each glial cell subtype exerts a different role within the CNS. As mentioned above, microglia represents the first line of CNS immunological defenses. After a lesion, in response to molecular signals (interferon γ, tumor necrosis factor α, macrophage colony stimulating factor, and granulocyte macrophage colony stimulating factor), microglial cells change morphology, passing from a branched phenotype to an early activated phenotype, or "primed" state, up to an actual active amoeboid phagocytic phenotype (Kreutzberg, 1996).

Astrocytes are not professional immune cells, but when activated can contribute to the local immune response. Once triggered after a brain injury, they become reactive and respond to pro-inflammatory

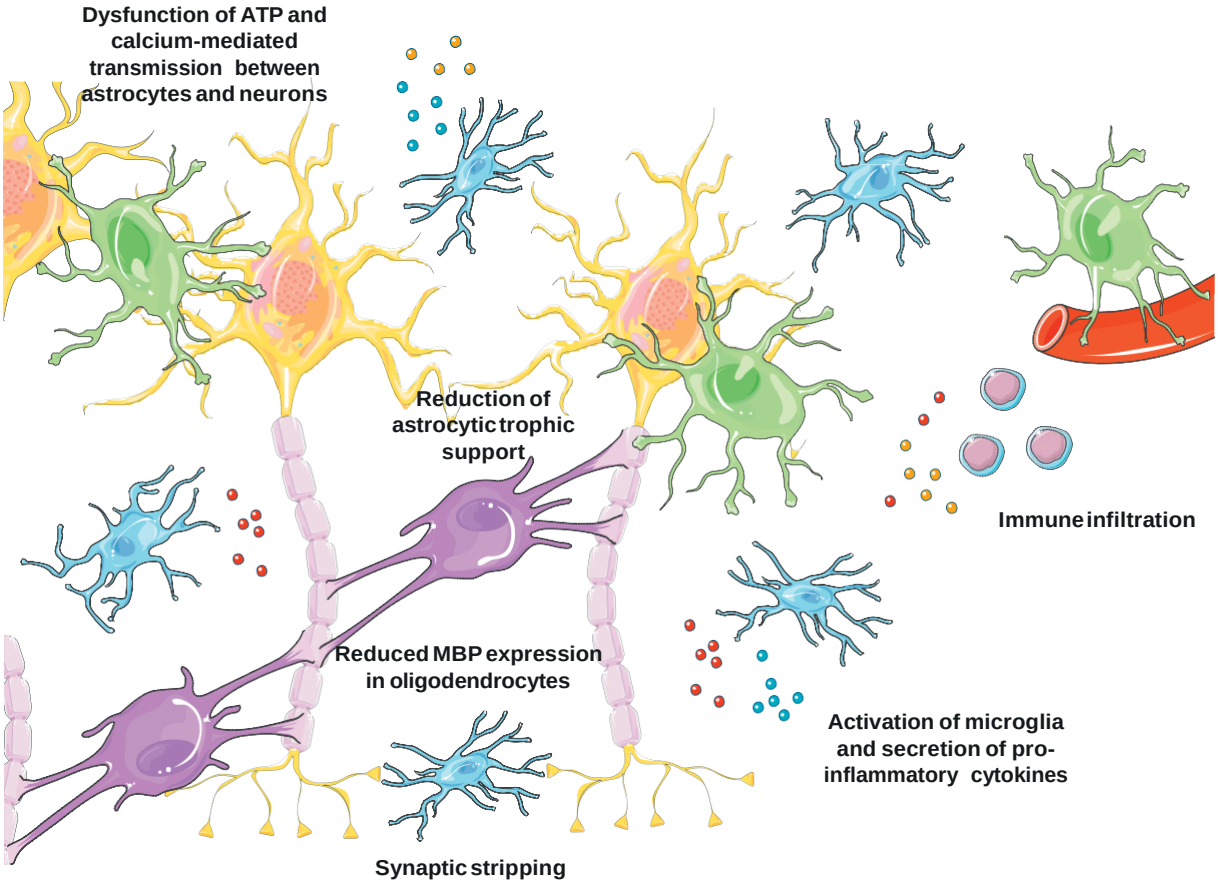

Fig. 1. The figure illustrates the pathogenic processes underlying SMA pathology in the CNS. Communication between astrocytes and neurons is impaired because of defect in ATP and calcium-mediated signaling. Similarly, structural dysfunction of astrocytes leads to decreased trophic support to motor neurons. An increase in the presence of activated M1 microglia is observed, with production of pro-inflammatory cytokines and activation of apoptotic cascade. Furthermore, activated microglia in SMA was shown to contribute to synapse loss through a process called synaptic stripping. The formation of a neurotoxic milieu causes the infiltration of immune cells which further reinforce microglial activation and neuroinflammation.

cytokines by proliferating and changing their morphology, growing numerous processes and developing hypertrophic nuclei (Pehar et al., 2005). Healthy astrocytes activated by peroxynitrite administration *in vitro* are able to promote apoptosis in previously healthy neighboring MNs through the release of ROS which in turn compromise the integrity of neighboring cells by damaging cell membrane proteins and lipids, as well as by oxidizing essential proteins for the MNs survival (Cassina et al., 2002).

From these studies, two main hypotheses for the sequence of events in glial response have emerged: a simultaneous activation hypothesis and a sequential activation one (Papadimitriou et al., 2010). The first hypothesis suggests that astrocytes and microglia react in concert to similar pathological signals that derive, for example, from compromised neighboring neurons. Conversely, the second hypothesis advocates that microglia and astrocytes could be able to influence each other: the activation of the microglia would determine increase of cytokines and NO, which can modulate astrocyte activation, and at the same time activated astrocytes would release cytokine and chemokines that activate microglia (Moisse and Strong, 2006; von Bernhardi and Eugenín, 2004).

Oligodendrocytes dysfunction has also been recognized in several neurodegenerative diseases, such as Parkinson's disease (PD) and Multiple System Atrophy. Indeed, close interconnection between oligodendrocytes and neurons is further proved by the fact that mice lacking the expression of specific oligodendrocyte markers develop axonal degeneration independent of demyelination (Griffiths et al., 1998; Lappe-Siefke et al., 2003). Among the hypothesized mechanisms, oligodendrocytes' dysfunction might cause neuronal death through the activation of neuroinflammatory mechanisms and the loss of neurotrophic support (Brudek et al., 2013; Fellner et al., 2013; Li et al., 2018; Ubhi et al., 2010; Zhang et al., 2005)

It remains to be clarified which signals trigger glial activation and which is the sequence in which the different inflammatory cells and mediators are recruited into play. To date, a convincing demonstration of such cell-cell interactions is still lacking, as contrasting data have been generated using different animal models.

6. Glial cells involvement and neuroinflammation in SMA

Some groups have shown that the loss of MN is detectable only in the final phase of the SMA (Kong et al., 2009; Ling et al., 2010). Before the loss of MNs, pre-synaptic defects appear, which include the loss of terminal arborization and the aggregation of intermediate filaments, which causes intermittent failures in neurotransmission (Kariya et al., 2008). Therefore, it can be deduced that the death of motoneurons is a late event in the pathogenesis of SMA, preceded by the activation of glial cells that could sustain and spread the degenerating process (Wang and Bordey, 2008). Indeed, in all types of human SMA, gliosis was reported to be associated with areas of motor neurons degeneration in the spinal cord and brain stem (Araki et al., 2003; Kuru et al., 2009). An important pathological feature of this disease is the glial bundle formation, especially in anterior roots and in the lumbar regions of the spinal cord, where motor neuron loss and glial activation are found (Ghatak, 1983). These bundles are thought to represent a protrusion of

reactive astrocytes into the neurilemmal tubes containing degenerating myelinated axons. In type II SMA, it was shown that gliosis in the spinal cord and in the brainstem is associated with a severe reduction of MN number, and that oxidative stress-related products are deposited into the remaining atrophic MNs (Araki et al., 2003).

As explained before, astrocytes and microglia activate in response to inflammatory stimuli and promote neuroinflammation, resulting in cytokines production, immune infiltration and activation of pro-apoptotic pathways. Traditionally, neuroinflammation was believed to be a secondary event, subsequent to neuron loss, and aimed at limiting cellular damages and at promoting CNS regeneration. However, as discussed below, this concept has been challenged by recent studies.

Thus, glial cells dysfunction is believed to promote the pathogenesis of SMA in several ways, which we are now going to illustrate (Fig. 1).

6.1. *Astrocytic dysfunction and defective astrocyte-neuron interaction*

Several findings support a contribution of astrocytes to SMA development. For instance prominent astrogliosis can be found at the level of the spinal cords in post-mortem SMA-1 patients, with an increase in GFAP-staining cells and in IL-6 and IL-1beta (Rindt et al., 2015).

SMN deficiency has been shown to impair normal astrocytic function in several ways (Table 1). Indeed, it has been observed that, during disease progression, astrocytes become reactive exhibiting both morphological and functional changes, and these activation-related morphological alterations appear long before the loss of the motor neurons (McGivern et al., 2013). McGivern and colleagues have recently shown that, starting from postnatal day 9, astrocytes in the spinal cord of a transgenic SMA murine model display an enlargement of cellular bodies, increased expression of GFAP in the cytoplasm and retracted processes (McGivern et al., 2013). These alterations were retrieved in induced pluripotent stem cell-derived SMA astrocytes as well. Both these findings seem to highlight that astrocytic dysfunction and loss in SMA might be a direct consequence of SMN deficiency, rather than of a diseased microenvironment. In the same study, a dysregulation of basal calcium and a reduction in the response to ATP stimulation were appreciated, thus suggesting a dysfunction of ATP and calcium-mediated transmission between astrocytes and neurons (McGivern et al., 2013; Parpura and Zorec, 2010). Another proof of a defective astrocyte-motor neuron communication comes from the observation that, in motoneuron–astrocyte contact cocultures, synapse formation and synaptic transmission were significantly reduced when either motoneurons, astrocytes or both were from SMA mice compared with wild-type cocultures (Zhou et al., 2016). Additionally, a downregulation of Ephrin B2, a membrane-binding ligand which regulates synapse formation, was retrieved in SMA astrocytes, thus suggesting a possible mechanisms linking astrocytes, motor neurons and the observed synaptic dysfunction. Moreover, astrocytes in SMA have been shown to contribute to motor neuron dysfunction also by decreased production of supportive factors such as monocyte chemoactive protein 1 (MCP1), a chemokine linked to neuroprotection from excitotoxicity, in cellular cultures (Martin et al., 2017). Its restoration reverted negative effects on motor neurons' bodies and neurites, thus correction of MCP1 deficiency early in the disease has been proposed by authors as a potential therapeutic approach to SMA. However, as MCP1 is also implicated in inflammatory process, its effects are not completely clear.

We have discussed above the potential role of motoneuronal-specific miRNAs alterations in SMA-related motoneuronal damage. Stemming from these observations, Sison and colleagues detected the upregulation of miR-146a in the secretome of SMA astrocytes (Sison et al., 2017). miR-146a is involved in modulating the immune system in response to interleukins and TNFα production. Furthermore, motor neurons loss induced by SMA astrocyte conditioned medium (ACM) is reverted by a miR-146a inhibitor. Potential downstream targets of miR-146a include downregulation of GDNF and GATA transcription factors, which are required for organogenesis.

The timing of astrocytic dysfunction and its temporal relationship to motoneuronal damage remain a matter of debate, although recent studies have challenged the traditional belief that it was a secondary event. It was observed that in both *in vitro* and *in vivo* SMA models there

Table 1
Studies regarding astrocytes' dysfunction in *in vivo* and *in vitro* SMA models.

Model	Relevant results	Ref
SMA iPSC-derived astrocytes and SMNΔ7-tg-mice spinal cord sections	- SMA astrocytes show signs of activation by characteristic morphological changes and up-regulation of GFAP and Nestin - Dysregulation of basal calcium and reduced response to ATP stimulation - Increased phospho-ERK1/2 expression and reduced GDNF production	McGivern et al., 2013
SMNΔ7-tg-mice treated with scAAV9-SMN(gfap); human SMA spinal cord sections.	- Astrogliosis was prominent in end-stage SMA mice and in post-mortem patient spinal cords - Restoring SMN in astrocytes improves lifespan and gross motor function and rescued defects in NMJs and proprioceptive synapses - Increased expression of proinflammatory cytokines was partially normalized in treated mice	Rindt et al., 2015
Spinal cord astrocytes and motoneurons primary cultures and cocultures from WT and SMNΔ7-tg-mice	- SMN deficiency in MNs impairs synapse formation and function - SMN deficiency in astrocytes alters calcium concentrations - SMN deficiency alters motoneuron–astrocyte interactions in synapse formation and function in contact cocultures - SMN deficiency alters Ephrin B2 expression in astrocytes	Zhou et al., 2016
Astrocytes and motoneurons cultures from spinal cord of WT and SMNΔ7-tg-mice and from control and SMA patients iPSCs	- SMA mice ACM provides decreased support of isolated MNs - SMA astrocytes produce decreased amounts of MCP1, and its supplementation improves motoneuronal growth	Martin et al., 2017
Astrocytes and motor neurons cultures from control and SMA patients-derived iPSCs	- increased production and secretion of miR-146a as compared to control in SMA astrocytes - Treating MNs with synthesized miR-146a molecules induces significant MNs loss - MNs loss induced by SMA ACM is blocked by a miR-146a inhibitor. - Downregulation of GDNF and GATA transcription factors	Sison et al., 2017
SMNΔ7-tg-mice spinal cord sections and SMA iPSCs-derived astrocytes	- Dysregulation of Notch signaling in the spinal cord in both early and late stages of SMA - Pharmacological inhibition of Notch signaling improved the motor functional deficits in SMA model mice	Ohuchi et al., 2019b

Abbreviations: ACM, astrocyte-conditioned medium; GDNF, glial derived neurotrophic factor; iPSC, induced pluripotent stem cell; MN, motor neuron; NMJ, neuromuscular junction; SMA, spinal muscular atrophy;SMN, survival motor neuron; tg, transgenic; WT, wild-type.

was an alteration of Notch signaling, a mechanism of juxtacrine communication between adjacent cells that promotes astrogenesis and astrocytic differentiation (Caraballo-Miralles et al., 2013; Ohuchi et al., 2019b). These findings suggest that chronic SMN depletion during developmental stages might act *via* Notch signaling dysregulation causing an abnormal increase in astrocytes in early stages. Furthermore, activation of astrocytes in SMA models seems to appear before motor neuron loss, as early as postnatal day 0–1, thus suggesting it could be a primary event in SMA pathogenesis (Dachs et al., 2011; McGivern et al., 2013).

Selective restoration of SMN protein levels in astrocytes by viral vectors was attempted by Rindt and colleagues, who found that it resulted in increased survival in intermediate and severe SMA models and in a partial normalization of the production of pro-inflammatory cytokines (Rindt et al., 2015). Notably, SMN restoration did not cause an increase in motor neuron numbers, but rather in the rescue of defects of neuromuscular junctions and proprioceptive synapses, suggesting that the observed treatment effects likely protected and functionally enhanced the remaining motor neurons.

6.2. Microglial involvement and synaptic remodeling in SMA

Not only astrocytes, but also microglia seem to be involved in SMA pathogenesis (Table 2). Recently, Tarabal and colleagues confirmed the presence of activated microglia in the lumbar enlargement at different disease stages in a rodent SMA model (Tarabal et al., 2014). In this model, the immunoreactivity for the specific microglial marker (Iba1) is significantly increased starting from the early symptomatic stages, surrounding the ventral horn motor neurons, thus suggesting the presence of active neuroinflammation.

In addition to that, they detected active microglial cells containing swallowed structural complexes, such as damaged presynaptic terminals and post-synaptic dendrites. The phagocytic function of microglia is an increasingly recognized phenomenon in many neurodegenerative diseases, and these results seem to underline the importance of the action of this type of cells in the elimination of cellular fragments during the degeneration of a synapse (Fu et al., 2014). These results confirm similar findings of a previous work by Ling and colleagues, which observed an increased colocalization of activated microglia with intense Iba1 immunoreactivity and MNs in the spinal cord, alongside with a reduction in central synapses, particularly glutamatergic ones, at the level of MNs (Ling et al., 2010). All these observations are in line with the hypothesis that microglia might contribute to synapse loss through a process called synaptic stripping, in which microglia, following insults in both central and peripheral nervous system, physically removes synaptic inputs from motor neurons (Perry and O'Connor, 2010).

A subsequent study further investigated activation of microglia in a murine SMA model, revealing that affected mice presented, in addition to microgliosis, a dramatic increase in harmful M1 microglia (Mac-2+) and a significant reduction in beneficial M2 cells (CD206+) in the spinal cord. Treatment with PRE-084, a sigma-1-receptor (Sig1R) activator, reduced the density of immunoreactive profiles and enhanced the number of CD206-immunolabeled cells found in murine spinal cord, although it was not able to prevent motor neurons loss nor to increase survival (Mancuso et al., 2012). This suggests a potential therapeutic effect of the activation of Sig1R, a transmembrane protein which seems to be implicated in in the protection of cells from both oxidative and endoplasmic reticulum-mediated cellular stress, in the reversal of M1/M2 balance disruption found in SMA.

Another recent study identified in the classical complement system the signal that initiate microglia-dependent circuits remodeling (Vukojicic et al., 2019). Authors found that C1q and C3, locally produced by microglial cells, tag vulnerable synapses in SMA which are later removed by microglia, and that SMN deficiency drives C1q tagging of proprioceptive synapses in the spinal cord. Postnatal block of C1q tagging with a monoclonal anti-Cq1 antibody was shown to result in synaptic and behavioral benefit in SMA mice. Furthermore, genetic deletion of C1q rescued synaptic deficit in SMN-deficient animals. This remarkable finding paves the way for further trials of complement inhibition in SMA models, and, perhaps, patients.

6.3. Downstream effects on pro-apoptotic/inflammatory pathways

Reactive astrocytes in SMA are able to trigger downstream inflammation and neuronal damage through the secretion of pro-inflammatory cytokines that can activate the apoptotic cascade. ERK 1–2, are members of the MAPK signaling pathway, with both a pro-survival and a pro-apoptotic action (Jo et al., 2005). One study showed that astrocytes derived from SMA IPSCs have an increased ERK1/2 phosphorylation, which could be a signal for activation of the apoptotic pathway through greater expression of pro-inflammatory and pro-apoptotic cytokines such as TNFα, IL-1 and IL-6 (McGivern et al., 2013). However, increased cellular death was not observed, nor it was increased secretion of TNFα, thus further studies using more sensitive detection methods are needed.

In addition to that, some of the deleterious effects linked to the glial reaction might depend on the activation of the apoptotic machinery

Table 2
Studies regarding microglial dysfunction in *in vivo* and *in vitro* SMA models.

Model	Relevant results	Ref
WT and SMNΔ7-tg-mice spinal cord sections	- Loss of central synapses with selective reduction of glutamatergic synapses onto L3–5 lateral spinal motoneurons - Reduction of proprioceptive sensory neurons - Increased association of MNs and microglia in the spinal cord	Ling et al., 2010
WT and SMNΔ7-tg-mice spinal cord sections	- Progressive MN size reduction and loss in the spinal cord of affected mice - Degenerating MNs show ultrastructural signs of degeneration (mitochondrial vacuolization, abnormal nuclei, dendritic degeneration) and mitochondrial depletion - Astroglial and microglial activation in ventral horns - defects in glutamatergic and GABAergic synaptic afferents	Tarabal et al., 2014
WT and SMNΔ7-tg-mice spinal cord sections	- Progressive astrogliosis and microgliosis around MNs - Increase of M1 microglia - Treatment with PRE-084 did not prevent MN loss but mitigated astrogliosis and microglia and induced M2 microglial phenotype	Cerverò et al., 2018
WT mice, SMNΔ7-tg-mice, $C1q^-$-tg-mice and SMNΔ7/$C1q^-$-tg-mice	- C1q and C3 tag vulnerable synapses in SMA mice, and microglia is responsible for synaptic removal - SMN deficiency drives C1q tagging of proprioceptive synapses - Block of C1q determines synaptic and behavioral improvement - Genetic deletion of C1q rescues proprioceptive synapses in SMA mice	Vukojicic et al., 2019

Abbreviations: MN, motor neuron; SMA, spinal muscular atrophy; SMN, survival motor neuron; tg, transgenic; WT, wild-type.

Table 3
Studies regarding Schwann cells' dysfunction in SMA models.

Model	Relevant results	Ref
WT and SMNΔ7-tg-mice	- capacity for synaptic sprouting following paralysis was reduced - rate of axon growth during nerve regeneration was unchanged - nerve-directed reorganisation of acetyl choline receptors was decreased - loss of terminal Schwann cells from the neuromuscular junction was observed	Murray et al., 2013
Schwann cells isolated from SMA mouse peripheral nerve	- widespread changes to the Schwann cell proteome, in particular regarding ubiquitination pathway - Pharmacological suppression of Uba1 in Schwann cells was sufficient to reproduce the defective myelination phenotype seen in SMA	Aghamaleky Sarvestany et al., 2014
Schwann cells primary cultures from WT and SMNΔ7-tg-mice and cocultures with purified DRG motor neuron from WT animals; Sections of intercostal and sciatic nerves from WT and SMNΔ7-tg-mice.	- Abnormal myelination of peripheral nerves in SMA mice - SMA-derived Schwann cells fail to respond normally to myelination cues - Increased apoptosis activation (cas-3) - Increased neuronal instability (reduced neurites density)	Hunter et al., 2014
WT and SMNΔ7-tg-mice treated with pMpz-SMN1 transgene	- Restoration of SMN protein levels restricted solely to Schwann cells reversed myelination defects, significantly improved neuromuscular function and ameliorated neuromuscular junction pathology in SMA mice. - restoration of SMN in Schwann cells had no impact on motor neuron loss	Hunter et al., 2016
WT and SMNΔ7-tg-mice	- NMJs of the diaphragm of SMA mice show a loss of synaptic vesicles and active zones - depletion of s100-positive perisynaptic Schwann cells	Neve et al., 2016

mediated by pro-inflammatory cytokines IL-6 and IL-8. Cardiotrophin-1 (CT-1) is a cytokine of the IL-6 family, which has been shown to delay axonal motor degeneration in mice with SOD1 overexpression (Bordet et al., 1999). The administration of CT-1-expressing adenoviral vectors in mouse model with neuron-restricted deletion of exon 7 resulted in an increase in survival and in an improvement of motor performances of the treated mice (Lesbordes et al., 2003). These results might therefore suggest that some cytokines could delay the characteristic degeneration in SMA.

Moreover, further studies have focused on the neuronal apoptosis inhibitory protein gene (NAIP), which localizes in the region adjacent to SMN1 and is frequently deleted together with SMN1 in 50% of SMA-1 cases and in 10–20% of SMA-2 and -3 cases, with a strong correlation between the deletion of NAIP and the severity of the disease (Roy et al., 1995). Furthermore, NAIP is also capable of interacting with TNF-α receptor-binding proteins (*e.g.* TRAF-1 and TRAF-2), blocking cell death, and its overexpression was shown to prevent cell death induced by NGF withdrawal or TNF-α receptor activation in neuronal precursors *in vitro* (Götz et al., 2000; Rothe et al., 1996).

TNF-α pathway is known to contribute to neuroinflammation through a number of different mechanisms (including regulating apoptosis). In SMA there seems to be an inverse relationship between the severity of the disease and the activation of the TNF-α pathway (Papadimitriou et al., 2010). In particular, in SMA-1, an important downregulation of P38 kinases, MAP kinase interacting serine / threonine kinase (MNK2) and IL-32 (a pro-inflammatory cytokine), which are induced in the TNF-α pathway; this result in the inhibition of protein synthesis and an alteration of the transcriptome with deregulation of genes involved in energy metabolism (Millino et al., 2009). Conversely, in SMA-3 muscles, increased levels of P38 kinase and a different transcriptional program, close to that found under normal conditions, were detected (Millino et al., 2009). Indeed, SMA-1 muscles appeared diffusely atrophic, while in SMA-3 samples the coexistence of atrophic and hypertrophic fibers could be appreciated. This findings might reflect the activation of different transcriptional pathways following down- or upregulation of TNF-α cascade in SMA-1 and SMA-3.

Activation of inflammatory pathways in SMA was further confirmed by Kim and colleagues, who reported that SMN depletion by RNA interference was able to potentiate IL-1β-induced IκB kinase (IKK) activation and the subsequent production of inflammatory mediators such as TNF-α and NO in the SMN-deficient microglia (Kim and Choi, 2017). Pro-inflammatory cytokines such as interleukin-1β (IL-1β) and TNF-α induce the NF-κB signaling pathway through the activation of TRAF6 and IκB kinase (IKK). Following these results, the authors speculated that SMN might function as an inhibitor of the NF-κB inflammatory signaling pathway in microglial cells, and its deficiency might instead trigger its activation.

As mentioned above, activation of inflammatory pathways within glial cells leads to a secondary motoneuronal damage *via* synaptic remodeling, induction of apoptosis and deprivation of trophic support. However, neuronal damage further reinforces these cascade generating a vicious cycle between glial cells and neurons. Indeed, subtle changes in the spinal microenvironment, elicited by compromised neurons, may be enough to activate neighboring glial cells. Researchers have demonstrated that motor neurons, stressed by trophic factor deprivation or by FAS ligation, respond by producing NO and superoxide (Estévez et al., 1998). This two substances can react with each other to form peroxynitrite, which can trigger reactive morphological changes and GFAP and inducible nitric oxide synthase (iNOS) expression in neighboring astrocytes (Cassina et al., 2002). Similarly, microglial cells can be activated *in vitro* by the supernatant from cultured MN-like clonal cell lines deprived of trophic support (He et al., 2002). Thus, intervening in these cycle by blocking the inflammatory cascade might have huge beneficial effects at both a neuronal and glial level.

6.4. Peripheral glial cells involvement in SMA

As mentioned above, Schwann cells are the principal glial cells of the peripheral nervous system (PNS). There are two types of Schwann cells, myelinating and non-myelinating; myelinating ones are responsible for the formation of the myelin sheath at the level of the motor and sensory neurons of the PNS (Chernousov et al., 2008).

It has been recently reported that low levels of SMN in Schwann cells are able to trigger changes leading to abnormal axonal myelination and to the interruption of the deposition of extracellular matrix (ECM) proteins in peripheral nerves (Hunter et al., 2014) (Table 3). In one study, Hunter and colleagues showed that Schwann cells isolated from SMA mice have reduced levels of SMN and don't respond to normal differentiation signals *in vitro* (Hunter et al., 2014). Moreover, they were not able to correctly myelinate healthy neurons in co-cultures, and exerted a damaging influence on neural stability. An activation of the apoptotic cascade within the cells was also detected. These effects did not seem to be related to intrinsic neuronal defects, but rather were likely due to Schwann cell-dependent defects in the ECM of nerves. The selective genetic correction of SMN levels in Schwann cells reverted myelination defects, improved neuromuscular function and

ameliorated neuromuscular junction pathology in SMA mice (Hunter et al., 2016).

These findings thereby suggest that SMN deficiency might induce primary, intrinsic changes in Schwann cells, with consequences for myelination, axo–glial interactions and development of the ECM in the peripheral nerve.

Schwann cells' defects appear to be able to induce pathology also at the level of the NMJ. A depletion of s100-positive perisynaptic Schwann cells was observed at that level of the NMJs in SMA mice (Neve et al., 2016). Murray and colleagues identified a reduction in NMJ remodeling in SMA animal models and a loss of terminal Schwann cells which could contribute to this defect (Murray et al., 2013).

Further studies have shown that Schwann cells in SMA models present different alterations at the level of growth pathways, cell death, survival and molecular transport. In particular, the ubiquitination pathways appeared to be disrupted in the Schwann cells lacking SMN (Aghamaleky Sarvestany et al., 2014). Furthermore, pharmacological suppression of an activator of the ubiquitin pathway in Schwann cells was sufficient to reproduce the defective myelination phenotype seen in SMA. Overall, these data highlight the important role of SMN in regulating the ubiquitination pathway and in maintaining Schwann cell homeostasis.

6.5. Oligodendrocytes involvement in SMA

The role of oligodendrocytes in the pathogenesis of Spinal Muscular Atrophy was traditionally believed to be scarce. In 2017, a study conducted on a transgenic SMA mice did not reveal differences in oligodendrocytes differentiation, morphology and myelinating properties compared to control (O'Meara et al., 2017). However, a recent report by Ohuchi and colleagues has challenged this view, describing defects in the differentiation and function of cells of the oligodendrocytic lineage derived from SMA mice and from SMA-patients iPSCs (Ohuchi et al., 2019a). In particular, expression of early (NG2) and late (MBP) markers of oligodendrocytes was reduced. Notch was involved in the decline of NG2 expression in the spinal cord of affected mice and, in addition, pharmacological Notch inhibition promoted differentiation of MBP-positive cells. Notably, oligodendrocytes' dysfunction appeared before motor neurons loss.

Further studies are needed in order to elucidate the potential role of oligodendrocytes in SMA pathogenesis. Nevertheless, the involvement of the oligodendrocytic lineage does not appear unlikely at the light of the numerous observations of a glial cells alteration in SMA.

7. Conclusions and future perspectives

From all the studies discussed so far, it emerges that SMA is a non-cell autonomous disease in which glial cells and motor neurons influence each other and contribute to denervation, synaptic loss and eventually cell death. Microglial cells and astroglial cell involvement has been widely investigated and is now increasingly recognized, while the role of peripheral Schwann cells is somewhat less delineated. Recently, a contribution of oligodendrocytes and of oligodendrocytes precursors has been revealed.

Several mechanisms seem to be implicated in this glial-mediated damage, and many are probably still obscure. Nonetheless, from our discussion it emerges as a prominent culprit the disruption in different neuronal-glial interactions, such as synapse formation and remodeling, intercellular transmission and myelination. Moreover, a relevant role is also played by the neuroinflammatory pathways, with production of pro-inflammatory cytokines, induction of apoptotic cascade both in neurons and glial cells and generation of a neurotoxic milieu with spreading of neurodegeneration. Novel clues may also arise from evidence concerning the role of peripheral adaptive immunity in the regulation of neuroinflammation in other neurodegenerative diseases, such as PD. T-lymphocytes were retrieved in psot-mortem brain specimens of animal models and PD patients (Brochard et al., 2009; McGeer et al., 1988), and an alteration in the circulating T-lymphocytes profile with a shift towards a “pro-inflammatory” Th1 pattern was detected (Kustrimovic et al., 2018). Surprisingly, this field is still relatively unexplored in SMA, although a recent study detected thymic architecture abnormalities, aberrant T-cell development and global cytokine dysregulation in SMA mice (Deguise et al., 2017). Thus, a role of adaptive immune system in SMA pathogenesis can be hypothesized and warrants further studies.

A deeper characterization of these processes in SMA is warranted, not only for a better understanding of the pathogenic mechanisms, but also for a refinement of our therapeutic weapons. In the last years, two novel therapies for SMA have been approved: *nusinersen*, an antisense oligonucleotide which favors the skipping of exon 7 of *SMN2* mRNA, thus determining the production of a functional protein, and *onasemnogene abeparvovec*, a gene therapy with AAV9 carrier which delivers an exogenous SMN cDNA copy. Furthermore, other therapies are currently being tested, such as *risdiplam*, an orally-delivered, splicing modifier small-molecule (Poirier et al., 2018). In this perspective, the contribution of glial cells to SMA raises some questions. First, the observation that glial cells dysfunction precedes motoneuronal loss *in vivo* should be taken into consideration for the definition of a therapeutic time window. Indeed, it could support the introduction of extensive newborn screening for *SMN1* exon 7 deletion, in order to target SMN deficiency in childhood- or adult-onset forms before it becomes clinically evident. Additionally, it is evident from the studies discussed above that restoration of SMN expression both in motor neurons and in astrocytes is necessary in order to exert the maximal effect on survival. However, data regarding penetration of the aforementioned therapies in glial cells are not yet available. Notably, experimental data regarding adeno-associated vectors showed that AAV9 proved able to preferentially transfect neurons in newborn mice and astrocytes in adult animals (Foust et al., 2009; Markakis et al., 2010). A further issue is represented by the involvement of peripheral nervous system, as several studies demonstrated an involvement of peripheral nerves, Schwann cells and neuromuscular junctions. Intrathecally-delivered therapies, such as *nusinersen* and other ASOs in general, are not able to reach this compartment, so it is possible that, with increasing use of this drug, we may witness the rise of new phenotypes dominated by peripheral and systemic symptoms. Surely, since it is now recognized that SMA is a multisystemic disease, rather than a motoneuronal one, the ideal disease-modifying drug should have the potential to target both the central (neurons/glial cells) and peripheral (axons/Schwann cells/peripheral organs) compartments. On the hand, each cell type may require different levels of SMN to function properly, thus the investigation of SMN levels regulation within different tissues and systems during development is warranted. The clarification of differential SMN expression during development and in different organs is important for the optimization of therapies not only for SMA-1 patients, but also for milder SMA forms, which seem to respond less optimally to CNS-directed SMN-restorative strategies, in part because the treatment is started later. Another interesting issue, as mentioned above, is the potential involvement of adaptive immune system, which, if confirmed, could pave the way for preclinical and clinical trials with immunomodulatory drugs.

Indeed, unraveling the molecular pathways that are affected by SMN deficiency also after the developmental phase, such as inflammatory pathways, glial-mediated immune activation and synaptic remodeling, may shed a light on the mechanisms of neurodegeneration in adult life and pave the way for novel therapeutic strategies.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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