**Summary.** Amyotrophic lateral sclerosis (ALS) is typically defined by a loss of motor neurons in the central nervous system. Accordingly, morphological analysis for decades considered motor neurons (in the cortex, brainstem and spinal cord) as the neuronal population selectively involved in ALS. Similarly, this was considered the pathological marker to score disease severity *ex vivo* both in patients and experimental models. However, the concept of non-autonomous motor neuron death was used recently to indicate the need for additional cell types to produce motor neuron death in ALS. This means that motor neuron loss occurs only when they are connected with other cell types. This concept originally emphasized the need for resident glia as well as non-resident inflammatory cells. Nowadays, the additional role of neurons other than motor neurons emerged in the scenario to induce non-autonomous motor neuron death. In fact, in ALS neurons diverse from motor neurons are involved. These cells play multiple roles in ALS: (i) they participate in the chain of events to produce motor neuron loss; (ii) they may even degenerate more than and before motor neurons.

In the present manuscript evidence about multi-neuronal involvement in ALS patients and experimental models is discussed. Specific sub-classes of neurons in the whole spinal cord are reported either to degenerate or to trigger neuronal degeneration, thus portraying ALS as a whole spinal cord disorder rather than a disease affecting motor neurons solely. This is associated with a novel concept in motor neuron disease which recruits abnormal mechanisms of cell to cell communication.

**Key words:** Non-autonomous cell death, Cholinergic partition cells, Renshaw cell, ALS spreading, Cell to cell propagation

**The concept of non-autonomous cell death**

Motor neuron disease is classically defined by the loss of motor neurons in the ventral spinal cord and brainstem along with degeneration of corticospinal and corticobulbar tracts. These neurons in healthy control are placed in the ventral part of the spinal cord within lamina IX and can be stained by using a variety of antigens such as Choline Acetyltransferase (ChAT, Fig. 1) or neurofilament protein SMI32 (Fig. 2). It is well known that a primary motor neuron cell culture carrying a mutation for ALS does not undergo neuronal degeneration in the absence of glial cells (Zhao et al., 2004). On the other hand, the addition of other cell types (glia) as occurring within a mixed primary cell culture from the spinal cord leads to neurodegeneration (Ferri et al., 2004; Di Giorgio et al., 2007; Ilieva et al., 2009). This means that in motor neuron disease, motor neurons *per se*, albeit carrying a fully penetrant gene mutation leading to familial (f)ALS, are not prone to die unless they are placed within a mixed cellular environment.
This fact leads to two main possibilities: (i) motor neuron disease is indeed a disorder involving cells other than motor neurons; (ii) other cell types are required in order to produce motor neuron loss in motor neuron disease (Fornai et al., 2008; Yamanaka et al., 2008). In fact, previous investigations showed a detrimental role for glial cells (Lobsiger and Cleveland, 2007; Yamanaka et al., 2008; Ilieva et al., 2009). Thus, a pure motor neuron culture needs the presence of glial cells or glia-derived soluble factors (cytokines) to degenerate. In an elegant study, the addition of astrocyte from inherited ALS patients to motor neurons in vitro was found to induce motor neuron degeneration (Meyer et al., 2014). This detrimental relationship does not require a physical contact between glial cells and motor neurons since it occurs also when motor neurons are separated from glial cells by adding the medium derived from ALS glia (Lobsiger and Cleveland, 2007). This indicates that soluble, low molecular weight compounds derived from glial cells are critical to trigger motor neuron death.

Further experiments carried out to identify these compounds pointed to TNF alpha and cytokines as powerful glia-derived trigger for motor neuron loss (Hemmer et al., 2001; He et al., 2002; Hensley et al., 2003; Ferri et al., 2004; Weydt et al., 2004). Later research demonstrated that TNF alpha per se cannot account for the massive extent of non-autonomous cell death. Therefore, other molecules were analyzed to portray comprehensively the occurrence of glia-induced non-autonomous cell death. These research efforts led to identify additional soluble factors such as TGFbeta1 and additional cytokines (Polazzi et al., 2009; Heneka et al., 2010; Endo et al., 2015). Further studies extended the role of glia-derived inflammatory cells to non-resident leukocytes as critical triggers for motor neuron disease (Beers et al., 2008; Chiu et al., 2008; Appel et al., 2010; Zhao et al., 2012; Henkel et al., 2013; Brohawn et al., 2016). Recently, original data indicate that the non-autonomous detrimental effects of astrocytes on motor neurons is grounded on the expression of MHC class I molecules on the surface of motor neurons making them susceptible to cell loss (Song et al., 2016). Conversely, the overexpression of MHCI on motor neurons membrane confers neuroprotection (Song et al., 2016).

In this way, circulating compounds including proteins and RNA within retromers may cross the blood brain barrier to interfere as immunological messengers in the process of neuronal degeneration (Unger et al., 1985; Freischmidt et al., 2015; Gambardella et al., 2016; Tasca et al., 2016). Nonetheless, the detrimental/beneficial effects of cells surrounding motor neurons may overcome the role of glia and/or other inflammatory cells. For instance, it looks like deleterious effects on motor neuron survival can be induced by neurons other than motor neurons. In fact, the role of glial cells despite not being clearly elucidated does not cover entirely the neuropathology of ALS since it is more and more evident that other neuronal cell types are involved. This is the case of neuronal cells other than motor neurons which disappear early in the spinal cord in the course of ALS (Oyanagi et al., 1989; Morrison et al., 1998).

**Pioneer and recent findings about neurons other than motor neurons**

The occurrence of widespread neuronal loss extending beyond the sole motor neurons in ALS was documented in human patients for decades (Oyanagi et al., 1989; Morrison et al., 1998). The occurrence of widespread neuronal loss extending beyond the sole motor neurons in ALS was documented in human patients for decades (Oyanagi et al., 1989; Morrison et al., 1998).
al., 1989, 1990; Hirano, 1991; Terao et al., 1994). The loss of neurons in wide areas of the spinal cord was described in both familial and sporadic (s)ALS encompassing Rexed’s laminae V, VI, VII, VIII and X. The spinal topography of neuronal loss led to include neurons other than motor neurons in the neuropathology of ALS (Morrison et al., 1998). This variety of spinal cord neurons may undergo cell death or slighter degenerative changes such as intracellular protein aggregates. For instance Pardo et al. (1995) found the occurrence of inclusions within neurons extending back to motor neuron nuclei. It is now evident that neuronal loss even in the anterior horn extends way beyond motor neurons of lamina IX. Despite providing a wider scenario to comprehend the neuropathology of ALS these findings also disclose a remarkable track to understand the molecular mechanisms of disease.

It was pioneer work carried out by Morrison and colleagues (1998) which broke the common belief of ALS as a selective degeneration of motor neurons. In fact, in their elegant study these Authors provided evidence for extensive neuronal loss encompassing a variety of neuronal pools in the whole anterior horn. Thus, the apparently novel concept of anterior horn disease as recently described (Pasquali et al., 2014) to mean more effectively what is considered by the term “motor neuron disease” was already seeded in Morrison’s work. Despite being missed out for almost two decades this work clearly indicates that a specific subtype of SOD1 mutation leads to a loss of more than 20% of total neurons counted in the anterior horn of the spinal cord. This count was astonishing for that time considering that 28% of motor neurons were lost in lamina IX only. Therefore, in order to keep high the percentage of neuronal loss in the whole anterior horn a considerable amount of neurons diverse from motor neurons were expected to die within ALS spinal cord (23.5%). These findings led Morrison et al. (1998) to conclude that “degeneration in the spinal cord of patients with ALS is not specifically directed at motor neurons, but rather more generally at several populations of neurons in the spinal cord”. Again, in this study a rough time course was carried out showing no selectivity for early compared with later stages in disease progression. In fact, the data reported above refer to a symptomatic disease stage, whereas during a pre-symptomatic (pre-motor) stage no significant reduction in any neuronal type was documented in Morrison’s original study. Thus, occurrence of cell death in multiple neuronal cell types led these authors to conclude that, in ALS neurodegeneration is not specific for motor neurons but instead motor neuron disease represents a disorder of multiple neuronal types. This is why in a recent work we re-defined motor neuron disease at the level of the spinal cord as an “anterior horn disorder” (Pasquali et al., 2014).

As introduced in the first paragraph of this manuscript, a plethora of neurons other than motor neurons of the spinal cord are involved in motor neurons disease. This evidence was developed progressively starting from the early works of Oyanagi et al. (1989) who noticed a massive loss of spinal interneurons. In

![Fig. 3. Double immunofluorescence in mouse ventral horn for glutamic acid decarboxylase (GAD67) and Calbindin D28K. Representative double immunofluorescence for glutamic acid decarboxylase (GAD67) and Calbindin D28K. When merging the staining co-localization is evident within motor neurons (small arrows) and Renshaw cells (large arrows) labelled with both antibodies. Cell nuclei are evident in blue with DAPI staining. Scale bar: 100 µm.](image-url)
The study by Oyanagi et al. (1989) demonstrated that in ALS patients there was a loss of interneurons specifically in the intermediate grey matter which added to the marked neuronal loss in the ventral part of the anterior horn. However, the severe loss of interneurons in the intermediate zone was not described to be severe in early disease stages when a marked motor neuron loss already occurred. Apart from multiple neuronal loss, Morrison et al. (1998) noticed that sub-cellular alterations such as the accumulation of phosphorylated neurofilamentous inclusions characterized both motor neuron and other neuronal cell types. In the work by Morrison et al. (1998) a variety of neuronal populations other than motor neurons were considered. Among these, the authors described a severe neuronal loss in the dorso-medial quadrant of the anterior horn (lamina VIII spreading over both lamina VII and lamina X). However, they did not provide specific phenotypes for these neuronal populations which were further dissected in subsequent studies.

Again, in their original manuscript Morrison et al. (1998) failed to document the time course of this multiple neuron loss which appeared simultaneous to their observations.

In keeping with this, most of those neurons other than motor neurons appear now to anticipate motor neuron loss. This is mostly evident for those neurons which are gifted with strong monosynaptic connections with motor neurons themselves. This is the case of Renshaw cells (Alvarez and Fyffe, 2007; Martin et al., 2007; Fornai et al., 2008; Wootz et al., 2013). These cells possess a double monosynaptic connection with motor neurons. They can be visualized as GAD67 and CalbindinD28K positive cells placed within lamina VII (Fig. 3) and they also contain gephyrin, thus they can be double stained with antibodies against Calbindin D28K and gephyrin (Fig. 4). These cells inhibit monosynaptically motor neurons. In fact as shown in Fig. 5 motor neurons which possess the enzyme Choline Acetyltransferase (ChAT) are double stained when exposed to primary antibodies against Calbindin D28K and ChAT. Renshaw cells in turn, are activated monosynaptically by motor neurons which may produce a double staining for Calbindin D28K and ChAT even on Renshaw cells (Fig. 5). These neuronal types may degenerate markedly and early in the course of the disease. For instance, Renshaw cell loss and/or a simple dysfunction of their glycinergic inhibition on motor neurons but motor neurons in ALS

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**Fig. 4.** Double immunofluorescence in mouse ventral horn for gephyrin and Calbindin D28K. Representative double immunofluorescence for gephyrin and Calbindin D28K. When merging the staining co-localization is evident within Renshaw cells (arrows) labelled with both antibodies. Cell nuclei are evident in blue with DAPI staining. Scale bar: 100 µm.

**Fig. 5.** Double immunofluorescence in mouse ventral horn for Choline Acetyltransferase (ChAT) and Calbindin D28K. Representative double immunofluorescence for Choline Acetyltransferase (ChAT) and Calbindin D28K. Renshaw cells (large arrows) are labelled only with Calbindin D28K antibodies. When merging the staining co-localization is evident within motoneurons (positive for both antibodies). Cell nuclei are evident in blue with DAPI staining. Scale bar: 100 µm.
neurons were described to occur more and before motor neuron loss (Martin et al., 2007; Fornai et al., 2008; Pasquali et al., 2009; Martin and Chang, 2011; Wootz et al., 2013; Ramírez-Jarquín et al., 2014; Siembab et al., 2016). Other cholinergic neurons of the spinal cord may degenerate in ALS. This is the case of cholinergic partition/commissural neurons in humans (Nagao et al., 1998) and experimental models (Casas et al., 2013; Fornai et al., 2014). This specific subset of spinal neurons is fundamental for producing locomotion (Miles et al., 2007; Zagoraiou et al., 2009). These neurons correspond to cholinergic cells belonging to lamina X which is markedly affected in ALS (Fornai et al., 2014). Further studies on partition cells demonstrated that they are mostly excitatory in nature and project both on motor neurons and Renshaw cells (Ramírez-Jarquín et al., 2014). This connection draws a sort of specific disease circuitry, which appears partly grounded on proprioception. In fact, despite being a motor disorder, ALS is now characterized by degeneration of sensory proprioceptive neurons in the spinal cord and brainstem as evidenced in a pioneering study and confirmed later on (Winder and Auer, 1989; Williams et al., 1990; Vaughan et al., 2015). Remarkably, proprioceptive neurons cover the origin of all main spino-cerebellar pathways. Thus, neurons of Clarke’s column, neurons of the lateral cuneate nucleus as well as spinal border cells and neurons of the interstitial cervical nucleus appear to be all involved in ALS (Murayama et al., 1989; Williams et al., 1990; Kokubo et al., 1999; Fujita et al., 2011). Evidence showing that proprioceptive impairment occurs early in ALS is provided (Lalancette-Hebert et al., 2016); in fact, sensory nerve endings within muscle, carrying action potentials from muscle spindles are severely affected as much as motor axons since the early stages of motor neuron disorders (Lalancette-Hebert et al., 2016). This wide scenario needs a detailed analysis of the specific roles of those neurons involved in motor neuron disease. Apart from proprioception, a loss of thin sensory afferents is described in ALS. This may relate to sensory alterations other than proprioception affecting Rexed’s lamina II and III known as substantia gelatinosa which possesses again a remarkable staining for Calbindin D28K as shown in Fig. 6. At any rate the proprioceptive alterations are the most remarkable in the sensory system of ALS patients.

Involvement of proprioceptive neurons in the loop of motor neuron degeneration

Functional and comparative neuroanatomy provided a great impulse to the concept of extended neuronal loss in motor neuron disease. Pioneer anatomical studies showed the basis for the concept of anterior horn disease (Morrison et al., 1998), while very recent anatomical evidence provided sound data showing the occurrence of specific neuronal cells apart from motor neurons in the course of motor neuron disease. This concerns a loss of the inhibitory effects of Renshaw cells on motor neurons (Wootz et al., 2013); a loss of cholinergic neurons placed in the dorso-medial extent of the anterior horn and lamina X (Morrison et al., 1998; Wootz et al., 2013). This adds to the early loss of proprioceptive neurons which form the spinocerebellar pathways (Murayama et al., 1989; Williams et al., 1990; Kokubo et al., 1999; Szaki et al., 2007; Fujita et al., 2011; Nakamura et al., 2014; Sabado et al., 2014). Thus, in keeping with the spinal cord, the disease extends beyond motor neuron-related circuitries to encompass more properly those neurons which are placed within proprioceptive loops including damage to the spinocerebellar pathways. This is consistent with damage to projecting neurons placed in the dorsal nucleus of Clarke, which gives rise to the dorsal spinocerebellar tract (Murayama et al., 1989; Kokubo et al., 1999; Fujita et al., 2011). Similarly, spinal border cells, which form both the ventral and rostral spinocerebellar tracts, are recruited in ALS (Williams et al., 1990; Nakamura et al., 2014). Again, damaged neurons selectively staining for 6A2 antigen corresponding to the origin of the cervico-spino-cerebellar tract are damaged in the central cervical nucleus (Williams et al., 1990). Thus, it is not surprising at all that cell loss extends to dorsal ganglia where ganglionic proprioceptive neurons may be affected when the disease extends to the dorsal column in humans (Szaki et al., 2007; Fujita et al., 2011; Sabado et al., 2014) and experimental models (Guo et al., 2009). This is in line with recent findings in humans and experimental models showing that both motor and sensory nerve fibers are affected (Vaughan et al., 2015; Natale et al., 2015; Schäfer et al., 2017) and both sensory and motor axons appear to be jammed by stagnant mitochondria within big autophagy vacuoles (Natale et al., 2015). In detail, proprioceptive axons, which innervate muscle spindles, are mostly affected, including both dynamic and static fibers (IA and II, respectively, Vaughan et al., 2015). In keeping with the concept of a disease recruiting

Fig. 6. Immunohistochemical localization of Calbindin D28K. Representative picture of immunohistochemical localization of Calbindin D28K in mouse dorsal horn (lamina 2 and lamina 3 forming the Rolando’s substantia gelatinsa). Scale bar: 100 µm.
proprioceptive loops, degeneration of interneurons in the spinal cord occurs for those cells which are excited by Ia and II fibers (Hongo et al., 1983). Remarkably, all targets of these proprioceptive fibers (alpha motor neurons, Clarke column neurons, spinal border cells, lateral cuneate nucleus, interstitial cervical nucleus, partition cells, Renshaw cells) are involved in neuronal degeneration. Thus, the scenario of extended neuronal loss within ALS spinal cord applies to neuronal circuits directly connected with motor neurons in the context of proprioceptive loops. The classic proprioceptive connection physiologically occurs during the so-called alpha-gamma co-activation. In this case, descending motor axons activate gamma motor neurons which promote activation of muscle spindles, which in turn excite Ia and II fibers leading to overactivation of alpha motor neurons. The very same proprioceptive fibers send axonal branching to Renshaw cells, partition cells and central pattern generators involved in locomotion (Ellaway et al., 2015). Remarkably, a recent experimental study demonstrates a marked discrepancy between the fate of gamma and alpha motor neurons in ALS, since the massive loss of alpha motor neurons is not matched by a similar finding for gamma motor neurons (Lalancette-Hebert et al., 2016). These authors found that gamma motor neurons are not simply spared but they rather exert a deleterious influence on motor neuron survival since the targeted suppression of gamma motor neurons protects alpha motor neurons from cell death (Lalancette-Hebert et al., 2016). This effect is bound to the overactivity of Ia fibers promoted by dynamic gamma motor neurons since the targeted reduction of Ia synapse over alpha motor neurons results again in neuroprotection (Lalancette-Hebert et al., 2016). These findings point out a putative novel source of degeneration in the pathophysiology of ALS and portrays the Ia fibers as the first step in driving neuronal degeneration along the various neuronal elements disseminated in the proprioceptive loop. In fact, gamma motor neurons lack Ia synapses, which are present on cholinergic partition cells, which are lost in ALS patients and are connected to motor neurons to generate locomotion (Miles et al., 2007; Zagoraiov et al., 2009). Similarly, Ia fibers target Renshaw cells (Siembab et al., 2016) as well as proprioceptive nuclei projecting to the cerebellum which receive specifically spindle afferents. These data highlight neurons involved in proprioception as a novel extended target of disease taking the place of the whole motor neuron population. This is in line with very recent findings showing the occurrence of altered sensory input in ALS (Dalla Bella et al., 2016; Sassone et al., 2016; Schäfer et al., 2017). In fact, ALS patients feature a massive deficit in proprioception due to the involvement of muscle sensory fibers before the deficit of motor innervation occurs. This is also evident in familial (f)ALS models featuring early loss of proprioceptive fibers which anticipates degeneration in the spinal cord (Vaughan et al., 2015). Such a deficit anticipate motor neuron loss and it is likely to be the real cause of impairment in the clasping reflex, which occurs early in the hind limbs of ALS models and was previously supposed to represent a motor deficit (Dupuis et al., 2009).

Concluding remarks

Current knowledge about cell-to-cell spreading of detrimental compounds as a key engine to promote disease progression leads to re-think about the significance of such an extended neuronal loss. When considering detrimental effects of glial cells one is forced to think about release of toxic cytokines as the molecular mechanisms which sustain the disease course. In contrast, when looking at the connectivity of neurons other than motor neurons, the scenario moves toward synaptic mechanisms of neurodegeneration. These two concepts are converging since recent studies demonstrated the pathophysiological consequence of immunosynapses (Rabinovich et al., 2005; Song et al., 2016). The progressive deciphering of degeneration occurring in specific sub-classes of neurons other than motor neurons in the spinal cord of both ALS patients and experimental models calls for identifying a proprioceptive unit which is primarily and entirely affected in motor neuron disease. This phenomenon recently appears to spare gamma motor neurons which instead may be the promoter of an altered proprioceptive activity which leads to early degeneration of afferents from muscle spindles to extend to their cell bodies in the dorsal ganglion projecting to ascending proprioceptive pathways, as well as spinal cord connected neurons, namely the Renshaw cells and commissural neurons. This proprioceptive chain of degenerative events along synaptically connected neurons may rely on the synaptic spreading of toxic proteins which was recently demonstrated in motor neuron disease (Fornai et al., 2011; Silani et al., 2011; Bretttschneider et al., 2014; Pasquali et al., 2014; Kassubek et al., 2014). In fact, both SOD1 and TDP43 which are pathologically accumulated within degenerating neurons are able to spread from cell to cell exploiting existing synaptic connections (Braak et al., 2010; Pasquali et al., 2010; Ferrucci et al., 2011; Fulceri et al., 2011; Lee and Kim, 2015; Feiler et al., 2015; Hasegawa et al., 2016; Jovičić et al., 2016; Braak et al., 2017; Nonaka and Hasegawa, 2017).

Acknowledgements. This work was supported by research grant PRIN 2010-2011 FF.

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Neurons but motor neurons in ALS

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Accepted April 1, 2017