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Review

Loss of neuromuscular junction integrity and muscle atrophy in skeletal muscle disuse

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ABSTRACT

Physical inactivity (PI) is a major risk factor of chronic diseases. A major aspect of PI is loss of muscle mass and strength. The latter phenomenon significantly impacts daily life and represent a major issue for global health. Understandably, skeletal muscle itself has been the major focus of studies aimed at understanding the mechanisms underlying loss of mass and strength. Relatively lesser attention has been given to the contribution of alterations in somatomotor control, despite the fact that these changes can start very early and can occur at multiple levels, from the cortex down to the neuromuscular junction (NMJ). It is well known that exposure to chronic inactivity or immobilization causes a disproportionate loss of force compared to muscle mass, i.e. a loss of specific or intrinsic whole muscle force. The latter phenomenon may be partially explained by the loss of specific force of individual muscle fibres, but several other players are very likely to contribute to such detrimental phenomenon. Irrespective of the length of the disuse period, the loss of force is, in fact, more than two-fold greater than that of muscle size. It is very likely that somatomotor alterations may contribute to this loss in intrinsic muscle force. Here we review evidence that alterations of one component of somatomotor control, namely the neuromuscular junction, occur in disuse. We also discuss some of the novel players in NMJ stability (e.g., homer, bassoon, pannexin) and the importance of new established and emerging molecular markers of neurodegenerative processes in humans such as agrin, neural-cell adhesion molecule and light-chain neurofilaments.

1. Introduction

Physical inactivity (PI) is a major risk factor of chronic diseases such as heart disease, diabetes and cancer (U.S. Department of Health and Human Services. 2008 Physical activity guidelines for Americans. www.health.gov/paguidelines/guidelines/, 2008). It is a frequent phenomenon that is observed in a variety of conditions such as immobilization following traumatic lesions, deconditioning, ageing, chronic diseases and bedridden patients. The recent COVID-19 confinement stressed its detrimental consequences (Narici et al., 2021). Although PI affects most organs and systems, the **loss of muscle mass and strength** is of paramount importance. It impairs everyday life and represents a major issue for global health impairing movement execution and exposing subjects to falls and fractures, favouring further deconditioning. Muscle loss is related to metabolic alterations, such as insulin resistance, anabolic resistance and to low-grade chronic inflammation, which are among the major risk factors of chronic diseases (Biolo et al., 2017; Bonaldo and Sandri, 2013; Booth et al., 2012; Mazzucco et al., 2010; Shur et al., 2022). In fact, muscle wasting worsens the prognosis of many chronic diseases and can be counteracted by exercise (Bonaldo and Sandri, 2013).

Muscle wasting develops very quickly. Data from short (3–14 days), mid (15–35 days) to long-term (36–120 days) bed rest indicate that **muscle atrophy** is 2–5% after 3–10 days of bed rest and ~ 10% after 7 days and it proceeds with a logarithmic time course, resulting in a loss of quadriceps muscle size of about 30% after 90–120 days (Marusic et al.,

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2021). The associated loss of muscle strength is even more striking as it is almost two-fold greater than that of muscle size (Marusic et al., 2021). Individual skeletal muscle fibres can show a significant decrease in specific force with disuse (Brocca et al., 2015; Hvid et al., 2017; Lamboley et al., 2016; Larsson et al., 1996). However, in muscle fibres, the reduction in myosin concentration and in the number of the actomyosin cross-bridges (Brocca et al., 2015; Hvid et al., 2017), the alterations in calcium (Ca^{2+}) dynamics (Monti et al., 2021) and possibly altered myosin function due to post-translational modifications (Li et al., 2015) are unlikely to fully account for the two fold difference between muscle mass and muscle force loss measured in vivo.

Importantly, alterations in **somatomotor control** could play a relevant role in causing a disproportionate loss of force compared to size observed with PI (Campbell et al., 2019; Clark et al., 2006). Indeed, inactivity-induced maladaptations have been found at all levels of the motor system, e.g., cortex, corticospinal excitability (Clark et al., 2008; Lundbye-Jensen and Nielsen, 2008b), motoneuron and NMJ function (Seki et al., 2001, 2007), possibly contributing to altered motor unit discharge (Seki et al., 2001). Among somatomotor alterations, **neuromuscular junction (NMJ)** instability (in which for instability we refer to any process underling detectable changing of the NMJ homeostasis) has been less investigated. This is especially true in humans due to difficulties in obtaining muscle samples containing NMJ. Hopefully, new techniques able to enrich muscle samples with NMJ will make such analyses easier (Aubertin-Leheudre et al., 2020).

In the scenario of loss of muscle mass and strength, aging and disuse share common features, such as altered mitochondrial dynamics, changes of morphology and function, and redox imbalance. These phenomena are commonly involved in the pathogenesis of muscle wasting, leading scientists to consider the exposition to microgravity as "a model of accelerated aging" (Biolo et al., 2003). Both phenomena can be involved in the pathogenesis of muscle wasting. Disuse is known to exacerbate the effects of aging on cells and tissues (Baehr et al., 2016; Hood et al., 2019). Interestingly, in murine models, aging per se causes NMJ instability and denervation of muscle fibres, ultimately causing muscle mass loss (Hepple and Rice, 2016). In contrast, such phenomenon seems to be controversial in humans, since studies by Jones et al., 2017 and Boehm et al. (2020a), (2020b) (Boehm, Miller et al., 2020; R. A. Jones et al., 2017) demonstrate that human NMJs are stable across the adult lifespan and in cancer cachexia, a strong muscle wasting disease. However, Jones et al., 2017 investigated a population of aged people with amputated leg (R. A. Jones et al., 2017) and Boehm et al. (2020a), (2020b) studied the rectus abdominis (Boehm, Miller et al., 2020), a muscle only partially involved in voluntary movements. Another study by Wokke et al., 1990 used the intercostal muscle (Wokke et al., 1990) reporting only ultrastructural changes of the NMJs. It is therefore clear that further studies on muscle directly involved in voluntary movements are required; where eventually the contribution of genetics in choosing the cohort of the study may be included (Sirago et al., 2022).

This review focus on the **potential role of NMJ alteration in disuse-induced skeletal muscle wasting**. We first review the emerging factors involved in NMJ stability. We will then consider disuse induced NMJ adaptations in murine and human models of disuse.

2. Emerging factors in NMJ stability and function

The NMJ is the site of communication between motoneurons and skeletal muscle fibres. Motoneurons rapidly transmit the impulse for contraction through the NMJ to target skeletal muscle fibres, where the contraction takes place. Upon the arrival of action potentials, the pre-synaptic motoneuron terminals release acetylcholine (ACh) in response to the influx of Ca^{2+} through voltage gated Ca^{2+} channels. ACh crosses the synaptic cleft and activates ACh receptors (AChRs) clustered on post-synaptic muscle fibres to depolarize the muscle cell and trigger Ca^{2+} release from the sarcoplasmic reticulum to initiate muscle contraction. This process takes the name of excitation-contraction coupling (ECC), in which several molecular players are involved (Table 1).

Table 1
List of the main biological players involved in the E-C coupling at the NMJ.

Biological factor	Localization	Role
Voltage dependent Ca^{2+} channels (VDCCs)	Pre-synaptic	Ca^{2+} entry to mediate ACh exocytosis
Bassoon	Pre-synaptic	Potentiation of VDCCs
Acetylcholine	Pre-synaptic & Synaptic cleft	Neurotransmitter
Agrin	Synaptic cleft	Activation of MuSK
Neurotrypsin	Synaptic cleft	Cleavage of agrin
Neural cell adhesion molecule (N-CAM)	Post-synaptic	Interaction with nerve during NMJ development
Muscle Specific Kinase (MuSK)	Post-synaptic	AChRs clusterization
Acetylcholine receptor (AChR)	Post-synaptic	Cations' entry to induce DHPR opening
Dihydropyridine receptor (DHPR or CaV1.1)	Post-synaptic	RyR opening
Ryanodine receptor (RyR)	Post-synaptic	Release of Ca^{2+} from SR
Na^+ and K^+ voltage gated channels	Post-synaptic	Generate Na^+ and K^+ currents
Pannexin	Post-synaptic	Potentiation of contraction
Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA)	Post-synaptic	Ca^{2+} re-uptake into SR
Calsequestrin	Post-synaptic	Binding of Ca^{2+} inside SR
Homer	Post-synaptic	NMJ relocation of NFATc
Nuclear factor of activated T-cells, cytoplasmic (NFATc)	Post-synaptic	Transcription coupling

The **synaptic cleft,** is estimated in a very large scale of about 30 – 100 nm in size, depending on the study (Nishimune and Shigemoto, 2018; Slater, 2017). It allows not only the transmission of the electric signal but may also have a direct role in mediating the rate of voluntary contraction through molecular effectors. In fact, acetyl-choline esterase (AChE) is present in excess at the synaptic cleft and hydrolyses most of the ACh released in quantal. This mechanism is adopted to finely control the chemical transmission and is preserved by the safety factor: an excess of ACh released at the synaptic cleft on every quantal release (Wood and Slater, 2001). Even though these excessive releases of ACh and AChE ensure to preserve the ECC, the size of the synaptic cleft may influence the timing of ACh migration in pathological contexts (Wood and Slater, 2001). The synaptic cleft (Fig. 1) is supported by the extracellular matrix (basal lamina), presenting a mixture of muscle and nerve-derived molecules that can modulate the electrical transmission. It contains collagens, fibronectins, nidogens, perlecan and different types of 'laminins chains' with a distinct pool for the NMJ site (Patton et al., 1997). There are important molecular effectors, such as pannexin, homer and bassoon, that clearly regulate the NMJ physiology, but their function is still not completely understood (Fig. 1). They are classified into categories depending on the localization (i.e., pre or post synaptic), and most of them seem to be involved in Ca^{2+} channels stabilization and ECC activation at both sides of the NMJ.

The **pre-synaptic** active zone-specific protein *bassoon*, usually recognized as scaffold protein, has been colocalized with the P/Q-type voltage-dependent Ca^{2+} channels (VDCCs) in animal models, promoting ACh release on the active zone (Chen et al., 2011; Nishimune et al., 2012). Such interaction seems to amplify Ca^{2+} influx and NMJ transmission at the nervous terminal and it may be retained to stabilize the NMJ site and enhance the ECC at the pre-synaptic side (Nishimune et al., 2012).

The **post-synaptic** zone is rich of AChRs and represents the terminal of the motor-neuron innervation. In humans, motor neurons innervate different types of fibres displaying marked differences in contractile and metabolic properties, the 'slow oxidative' type I, the 'fast oxidative/glycolytic' type IIA and the "fast glycolytic" type IIX. Hybrid fibres (type I-IIA and type IIAX) exist and can be very frequent (Schiaffino and Reggiani, 2011). It has been known for a long time that, among elite

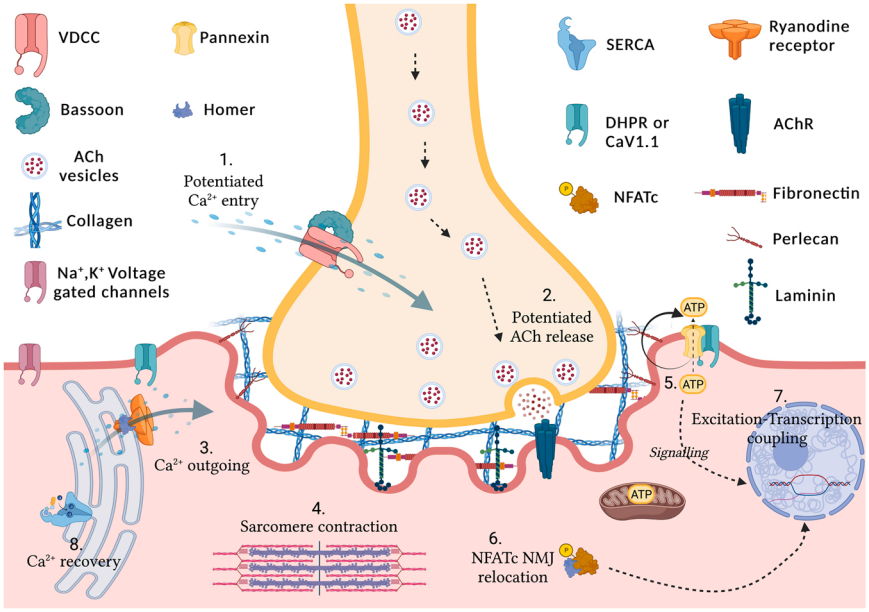

Fig. 1. Emerging factors involved in the E-C coupling events at the NMJ.
Excitation-contraction coupling (ECC) events take place at the neuromuscular junction (NMJ); where bassoon may be implicated in the potentiation of the first calcium entry, responsible of the acetylcholine (ACh) release through exocytosis events (1−2). Then the conformational changes on the acetylcholine receptors (AChRs), induced by ACh, give rise to post-synaptic events that lastly bring to the release of Ca^{2+} from sarcoplasmic reticulum (3), where homer may potentiate the release. Na^{+} and K^{+} voltage gated channels participate in the generation of the ECC, generating Na^{+} and K^{+} currents. The contraction of sarcomeres (4) and the release of adenosine triphosphate (ATP) by mitochondria, allow to the multiprotein complex of pannexin placed at the beginning of the T-tubule membrane (5), to release ATP in the extracellular matrix and activate the signalling on the NMJ microdomain. This excitation-transcription coupling signalling can also involve the nuclear factor of activated T-cells, cytoplasmic (NFATc) (6), relocated by homer at the NMJ microdomain and lastly promote the protein synthesis, muscle growth and switching of fibres (7). In the end, Ca^{2+} is recovered from the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump in the sarcoplasmic reticulum (SR) (8).

athletes, sprinters have higher proportions of fast fibres and endurance trained athletes have higher proportions of slow fibres (Costill et al., 1976). Interestingly, the size of the NMJ endplate is different between type I and II, being larger in type II than I, in which the contraction event requires high ECC in a short period of time (Ogata, 1988; Mantilla et al., 2007), even though some controversial exists since the diaphragm of adult male C57BL/6 J mice shows no NMJ differences between type I and II fibres (Sieck et al., 2012). Among the post-synaptic proteins, the *homer* family proteins are constitutively expressed in skeletal muscle (Salanova et al., 2011; Shiraishi-Yamaguchi and Furuichi, 2007; Soloviev et al., 2000). The function of the homer proteins in skeletal muscle is still unknown, even though it seems to act as scaffold protein involved in the intracellular Ca^{2+} signalling pathway activated by ECC, at the NMJ subsynaptic domain. In fact, both in vitro and in animal models evidences support that homer physically interacts with ryanodine receptor 1 (RyR1) on the junctional sarcoplasmic reticulum (SR), enhancing its activity and maybe mediating the interaction with other players, such as the triad transmembrane protein dihydropyridine receptor (DHPR or CaV1.1) that enhances ECC (Feng et al., 2002; Nakai et al., 1996; Schredelseker et al., 2005; Stiber et al., 2005).

Among the proteins able to interact with the triad's transmembrane proteins RyR-DHPR, there are also *pannexin* hemichannels (Arias-Calderón et al., 2016). These proteins act as unselective channels but have been also implicated in the activation of the excitation-transcription coupling (ETC). ETC allows the transcription of genes expressed in response to the ECC, through the interaction with the voltage sensor DHPR (or CaV1.1); forming a big multiprotein complex, as shown in murine models (Arias-Calderón et al., 2016; Jaque-Fernández et al., 2021).

Generally, such ancillary proteins act at different levels of regulation interacting also with signalling pathways. For example, homer isoforms interact with the nuclear factor of activated t-cells (NFATc). NFATc proteins are responsible of the activation of slow-twitch genes during exercise, to promote the switching of muscle fibres (Chin et al., 1998; Kubis et al., 2003). Calcineurin is the calcium-regulated serine/threonine phosphatase that responds to the intracellular Ca^{2+} increase during ECC and promotes NFATc nuclear translocation, by its dephosphorylation (Chin et al., 1998; Kubis et al., 2003). Interestingly, in humans it has been shown how the isoform homer2 may sequester and relocate NFATc1, the inducible component of the nuclear NFATc complex, relocating it to the NMJ site during the ECC events and resulting in protein synthesis (Salanova et al., 2011; Tothova et al., 2006).

2.1. Agrin

Agrin is a proteoglycan with fundamental roles in NMJ formation and maintenance. Agrin (from the Greek 'agrein'=to accumulate) is a heparan sulphate proteoglycan isolated for the first time from the electric organ of *Torpedo californica,* and characterized by AChR-aggregating properties (Nitkin et al., 1987; Wallace, 1989). Since the 'agrin hypothesis' has been postulated by McMahan in 1990, the involvement and the central role as motor-neuron derived proteoglycan, located in the synaptic cleft and supporting NMJ formation, clearly emerged (Kleiman and Reichardt, 1996; McMahan, 1990). Today, agrin is recognized as mediating and favouring not only NMJ formation in skeletal muscle, but also synapses in brain and immunological synapses in immune cells, showing how such versatile big molecule of ~600 kDa can dispatch several functions in humans, also in the absence of a proper basal lamina (A. A. Khan et al., 2001). Agrin is subjected to several alternative splicing that affects localization, binding to different substrates and function. In general, the main regions are the 'signal sequence' (SS) and the 'amino (N)- terminal domain' (NtA): the SS mediates its release in the synaptic cleft, whereas the NtA allows its binding to the laminins of basal lamina. The third segment, called 'type II transmembrane segment' (TM) allows the anchoring of agrin to the extracellular space in the brain, where a proper basal lamina is absent. In the amino-terminal region is located the N-CAM binding site, whereas in the carboxy-terminal there is the essential 95 kDa AChRs-aggregating region.

Differently from the muscle isoform, the neural isoform of agrin derived from motor-neuron is unique, because it retains the AChRs-aggregating properties, which takes place through the activation of the low-density lipoprotein receptor protein 4 (Lrp4) - muscle specific kinase (MuSK) axis (Bezakova et al., 2001; Zhang et al., 2011; Zong et al., 2012). Lrp4 belongs to the low-density lipoprotein receptor protein family and is expressed on the surface of skeletal muscle, where it acts as coreceptor to mediate the tetramerization of agrin with MuSK, essential for its activation (Zong et al., 2012). MuSK is the receptor tyrosine kinase selectively expressed in skeletal muscle that undergoes autophosphorylation, which is recognized by an adapter protein called docking protein 7 (Dok7) in a process crucial for MuSK activation. MuSK

activation guides the NMJ formation and AChR clustering on the endplate, where AChRs show high metabolic stability with an half-life of about 13 days which can be altered by stimuli, such as fasting or E3 ubiquitin-protein ligase MuRF-1 overexpression, due to the intervention of the autophagic system (Baehr et al., 2021; Khan et al., 2014; Rudolf et al., 2014). MuSK, Lrp4 and Dok7 alongside AChRs are pre-clustered on sarcolemma in structures called lipid rafts. Upon activation with agrin, released by the motor-neuron, they activate the innervation gene program in muscle that allows successful interaction between muscle and nerve (Bergamin et al., 2010; Till et al., 2002; Watty et al., 2000). The innervation gene program includes a retrograde signal under the control of MuSK, which causes a gradual decrease of N-CAM preventing hyperinnervation (Till et al., 2002).

2.2. Neural-cell adhesion molecule

Neural-cell adhesion molecule (N-CAM) is a glycoprotein involved in NMJ formation during embryogenesis, and in adult reinnervation processes. N-CAM belongs to a family of transmembrane glycoproteins with several isoforms that originate from alternative splicing and are delivered into the cell membrane, where they act mainly to mediate heterotopic and homotropic cell-adhesion interactions. In the latest 80's, N-CAM has been shown to be expressed and located on human myoblasts and myotubes in culture, but to be absent in adult skeletal muscle cryostat sections (Moore and Walsh, 1985; Walsh and Moore, 1985). Furthermore, evidence and data reported in mouse muscle showed that it is expressed during developmental embryogenesis in skeletal muscle and is associated to innervation, as well as reinnervation processes. N-CAM protein appears on junctional sarcolemma at the NMJ, intramuscular nerves, satellite cells and nodes of Ranvier (Cashman et al., 1987; François Rieger et al., 1985). N-CAM protein seems to mediate also the contact and maybe the entire enveloping process of Schwann cells, and the deposition of collagen fibrils in the extracellular matrix (Cashman et al., 1987; F. Rieger et al., 1988; François Rieger et al., 1985). Thus, N-CAM protein seems to allow the recognition and interaction of skeletal muscle fibres by the innervating neuron at the NMJ, allowing complete innervation. Its disappearance in adulthood could be instrumental in limiting hyper-innervation. However, the exact molecular events that regulate the expression of N-CAM in skeletal muscle and guide the innervating process, are still unknown. The existence of a soluble N-CAM form detected in conditioned media harvested from muscle cells has been reported in the past (Gower et al., 1988). However, it is still unknown if it is generated from sarcoplasm, or cleaved outside by proteases to mediate cell adhesion, or with other unidentified functions. Studies on N-CAM derived from cancer cells have associated secreted and serum N-CAM to disease activity in humans, suggesting a potential use for diagnosis and prognosis (De Jong et al., 1999; Jaques et al., 1993). In fact, N-CAM belongs to the key members of immunoglobulin-like CAM (Ig-CAM) family involved in cell-cell and cell-extracellular matrix adhesion processes in innate immunity. These classes of molecules have affinity also for β-integrin that activates the intracellular cascades through focal adhesion kinase (FAK), which undergoes phosphorylation in neurons. The FAK – extracellular signal-regulated kinase (ERK) – nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) axis is then responsible for modulating gene expression of neural receptors and channels (Colombo and Meldolesi, 2015), explaining one possible mechanism by which N-CAM may mediate muscle-nerve interaction. N-CAM has been extensively studied in the nervous system, cancer invasion and innate immune system. It is also named CD56, frequently used as phenotypic marker to distinguish different subsets of natural killer cells (NK cells) and their degree of activation in innate immunity. N-CAM protein is also expressed in plasmacytoid and myeloid dendritic cells (DCs) and their renewal partners, the monocytes, resulting involved also in adaptive immunity. The expression and upregulation of immune receptors, such as CD56, in skeletal muscle as happens in immune system, points to an emerging role of skeletal muscle as endocrine organ. Indeed, skeletal muscle can regulate metabolic states in the entire body through the expression of immune receptors and release of myokines (Pillon and Krook, 2017; Reidy et al., 2019; Whitham and Febbraio, 2016). However, the presence of the N-CAM binding site in agrin and the fundamental role of both molecules to instruct, guide and halts the embryological innervation and reinnervation process after injury and in adult, may suggest not only the reciprocal interaction, but also interaction with components of the extracellular matrix.

Interestingly, the presence of injury promotes the expression of marker molecules typically expressed during neuro-muscular embryogenesis, in which NMJ and muscle physiology is not established, is weak and requires a developmental program to be activated. Among such factors proposed in literature there are, agrin, N-CAM and recently the hemichannels (discussed more ahead) which may be promoting changes in cell communication signal, spatial expansion, maturation and membrane potential.

2.3. Myokines

The process of ECC has been also associated with the production and release of myokines from skeletal muscle (Pedersen et al., 2007; Severinsen and Pedersen, 2020), also through the release of extracellular vesicle (EVs) (Whitham et al., 2018). EVs have been proposed as vehicles of metabolic enzymes, lipids and microRNAs (called myomiRs) with specific functions for organs intra- and inter-communication (Luu et al., 2020; Whitham et al., 2018; Whitham and Febbraio, 2016). Myokines are known to exert the beneficial effects of exercise at systemic level and target different organs (Pedersen et al., 2007; Severinsen and Pedersen, 2020). They are major contributors to the intricate, complex, but beneficial communication between the brain and skeletal muscle through the NMJ. There are also myokines proposed to be beneficial for NMJ stability through the retrograde signal, such as neurotrophins, myotrophins such as the insulin-like growth factor 1 (IGF-1) (Salanova et al., 2014), and microRNAs.

Neurotrophins are specific neural factors, which are released at systemic level but act also locally at the NMJs' sites. They include soluble factors that control synaptic plasticity, namely *neural growth factor* (NGF), *brain-derived neurotrophic factor* (BDNF), *glial-cell-line-derived neurotrophic factor* (GDNF), *neurturin* (NRTN), *neuregulin-1* (NRG1), *neurotrophin-3* (NT-3) and *neurotrophin-4/5* (NT-4/5). Some of them have been recently characterized in vitro to be responsible to form a microenvironment favourable to NMJ maturation (Saini et al., 2021). NRG1, NT-4 and BDNF are able to maintain optimal neuromuscular transmission, as reported in animal models (Funakoshi et al., 1995; Handschin et al., 2007; Lebrasseur et al., 2003; Mantilla et al., 2004; Sandrock et al., 1997). In particular, NRG1 supports and maintains mature AChR pool at the NMJ, in a peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α) dependent-manner, promoting better performance during the ECC event (Handschin et al., 2007; Lebrasseur et al., 2003; Sandrock et al., 1997).

MicroRNAs have a direct role in NMJ homeostasis, highlighted by its direct influence on the gene regulation of hemichannels during embryogenesis. Hemichannels are unselective membrane channels that can regulate membrane potential at rest and during ECC, as well as the extrusion of molecules such as adenosine triphosphate (ATP) (Cisterna et al., 2014). Thus, they regulate important physiological functions and participate with autocrine properties in muscle homeostasis during embryogenesis. Connexin 43 (Cx43) is among the more important and studied isoform of hemichannels in skeletal muscle. During development, the human miR-206 downregulates Cx43 expression to promote correct neuronal control after birth, establishing a negative feedback loop with myogenin (Anderson et al., 2006; Moresi et al., 2010a; Valdez et al., 2014; Williams et al., 2009). In fact, the expression of connexins seems to reduce and/or stop with the increment of neuromuscular activity after birth. The nerve excision in animal models seems to revert

such scenario, going back to patterns expressed during development (Cea et al., 2013; Cisterna et al., 2014, 2016, 2020).

3. NMJ in disuse

The embryonic development of skeletal muscle starts in the absence of neural influence. Its subsequent growth and survival depends on motor innervation as described decades ago, and requires a very complex interchange of signals between muscles and nerves (Buffelli et al., 2018; Harris, 1981). As reported in murine embryogenesis, innervation controls at the transcriptional level the accumulation of important products (e.g. myosin alkali light chain, MLC; myogenic regulatory factor, MRF) that drive not only muscle development, but also genesis of the specific muscle types (Washabaugh et al., 1998, 2007). The *bidirectional signalling* between muscle fibres and nervous cells is at the base of muscle contraction and allows to maintain the correct muscle function.

For ethical and technical reasons, most research on the role of the NMJ in disuse-atrophy is focused on animal models. The existence of shared molecular pathways between humans and rodents is well known. Rodents proved to be fundamental models for the understanding of the biology of the NMJ, of new orphan proteins and for identifying new biomarkers of disuse. However, as the response to disuse has been shown to vary among species (Pellegrino et al., 2011), perhaps mainly due to morphological differences of the NMJ (Boehm et al., 2020a, 2020b), human studies are essential. Moreover they enable to better comprehend individual differences (Sirago et al., 2022).

3.1. Electrical activity controls spatial organization

Electromyogram (EMG) activity disappears and motor nerve activity is reduced on neurogram immediately after hindlimb unloading (HU) (De-Doncker et al., 2005), and potentially altering NMJ homeostasis. Not surprisingly, NMJ shows molecular and morphological remodelling following a change in electrical activity, i.e. exercise (Deschenes et al., 2019; Nishimune and Stanford JA, 2014) and disuse (Deschenes MR et al., 2006), which have an opposite effect.

Electrical muscle activity controls also the spatial expression and organization of important molecular players, such as AChRs. In fact, in animal model the denervation releases the inhibition of AChRs expression by extrajunctional myonuclei, causing AChRs expression all along muscle fibres (Buffelli et al., 2018). This indicates that a constant electrical activity is essential to maintain a proper molecular pattern along the sarcolemma, and that it is loosed when disuse stops the electrical transmission. Also Deschenes et al. (2006) showed that in 7 weeks old rats, 10 days of HU affect several parameters of post-synaptic NMJ morphology indicating reduced endplate dimensions, whereas do not cause significant pre-synaptic adaptations (Deschenes et al., 2006). Thus, the muscle side could be more sensible especially at the beginning of disuse and at least in aged rodent models. In fact, changes in expression of several molecular markers of the NMJ instability among which transcripts of AChRs isoforms α, γ, δ and ε, were observed following 14 days of HU in old (29-month-old) but not in adult (9-month-old) rats (Baehr et al., 2016). Collectively, such data suggest that NMJ remodelling following disuse might vary with the age of animals, the duration of the intervention and the time after HU when the NMJ is studied.

3.2. Early molecular events taking place during disuse

The time-course of the events taking place since the disuse begins is still under research. Recent studies on mice showed that, whereas disuse induced phenotypic changes, such as muscle atrophy, are progressively established in a time frame of weeks HU (e.g., 7–14 days), the underlying molecular phenomena, such as enhanced atrogenes expression, occur much earlier and being transient, die away by the time atrophy is established (Brocca et al., 2022). Consequently, a time-course analysis of the phenomena has been suggested as the ideal approach to study the pathogenesis of disuse adaptations (Brocca et al., 2022).

Notably, a recent study in a murine model showed that NMJ adaptations determining muscle atrophy can occur even within few hours of HU. Indeed, Chibalin et al. (2018) showed NMJ morphological adaptations, such as decreased endplate area occurring within 6–12 h after HU in rat soleus muscle (Chibalin et al., 2018). In the same muscle and at the same time, activation of several kinases, i.e., protein kinase C and A (PKC; PKA) and phosphorylated extracellularly regulated kinases 1/2 mitogen-activated protein (pERK1/2 MAP) kinase were enhanced; whereas activation of AMPK and its substrate acetyl-CoA carboxylase (ACC) was lower, accompanied by autophagy activation (Chibalin et al., 2018). This suggests that at the beginning of disuse, profound metabolic changes due to phosphoproteome alterations and removal of organelle components may occur, lastly influencing NMJ homeostasis.

The composition of the sarcolemma has a direct role in stabilizing the NMJ molecular players. Lipid rafts, i.e, cholesterol-rich plasma membrane lipid microdomains, were shown to play a key role in stabilizing post-synaptic NMJ anchoring AChRs, rapsyn, MuSK and Src-family kinases (Willmann et al., 2006). Interestingly, lipid rafts disturbance and inhibition of Na-K-ATPase α2 isozyme were also observed 6–12 h after HU in rodent model and were reversed by cholesterol supplementation (Kravtsova et al., 2016; Petrov et al., 2017). In the same model and at the same very early time, the content in ceramide, a signalling lipid involved in lipid rafts stability, increased in the sarcoplasm and at both junctional and extra-junctional compartments possibly supporting lipid raft disturbances and profound membrane changes (Petrov et al., 2019). Thus, the composition of the sarcolemma is among the first myocellular events to change with disuse, possibly to adapt skeletal muscle fibres' physiology to different stimuli and facilitate the relocation of molecular players.

The relocation of NMJ molecular players, such as MuRF-1, an ubiquitin ligase whose up-regulation plays a major role in disuse-induced muscle atrophy, can influence these profound changes. MuRF-1 was shown to be highly enriched at the NMJ, to colocalize with AChRs and to directly control NMJ stability through a transcriptional modulation of growth and DNA damage-inducible A (Gadd45a) and AChRα1 genes in animal model (Baehr et al., 2021; Rudolf et al., 2013). Also, MuRF-1 has been shown to be up-regulated within one day of HU in mice (Brocca et al., 2022). It could, therefore, play a role in disuse induced NMJ remodelling too.

Collectively these findings suggest a tentative, but intriguing hypothesis in which a very early decrease in the AMP-activated protein kinase (AMPK) activity, induced by a decrease in energy demand of suddenly inactive muscle fibres, could trigger: (i) NMJ remodelling through alterations of lipid rafts, AChRs clustering and Na-K ATPase function (which were shown to co-localize (Chibalin et al., 2018)); (ii) early onset of insulin resistance, through altered glucose transport and cholesterol toxicity; (iii) decrease in PGC1α expression. In turn, decreased PGC1α would (i) alter expression of key components of the NMJ contributing to NMJ instability, (ii) alter mitochondrial biogenesis and dynamics and oxidative metabolism, (iii) disinhibit forkhead box protein O (FoxO) dependent atrogenes expression, a major phenomenon of disuse muscle atrophy, (iv) activate inflammatory myokines, which would inhibit β-cell insulin secretion. Altered mitochondrial dynamics would likely cause (i) impaired energy metabolism stimulating a later AMPK activation, which would further stimulating atrogenes activation, (ii) reactive oxygen species (ROS) production, which would cause oxidation of proteins and functional impairment and activation of serine kinases, leading to phosphorylation of the insulin receptor substrate (IRS), thereby sustaining insulin resistance (Mazzucco et al., 2010).

3.3. Emerging NMJ players in disuse and new hypothesis

While the effects of disuse on skeletal muscle becomes clearer at

molecular level, new molecular players are studied and proposed for possible pharmacological treatments. MicroRNAs are particularly studied for their involvement in disuse. They are emerging as pervasive gene-expression regulators, at both systemic and local level. The presence of microRNAs, as well as long non-coding RNAs (lncRNAs), concentrated close to NMJ, have been associated to reinnervation processes in disuse animal models (Hitachi et al., 2020; Valdez et al., 2014). MicroRNAs can also control hemichannels expression during development, as mentioned above. The inhibition of connexins' hemichannels can also prevents the denervation related atrophy, as shown in the same study (Cea et al., 2013). This may open new hypotheses on the potential role of hemichannels expression in disuse and if it relates with NMJ homeostasis or not. Further studies are needed to clarify if denervation in murine models, among which nerve damage or excision, can share some molecular patterns with the denervation events observed in human models of disuse, as discussed in the next section.

3.4. Disuse-mediated denervation events and NMJ instability

In humans, disuse-induced adaptations are observed not only at muscular, but also at multiple levels of somatomotor control. Motor cortex functional connectivity (Newbold et al., 2020), motor behaviour and coordination (Moisello et al., 2008), motor evoked potentials (MEP) (Clark et al., 2008; Lundbye-Jensen and Nielsen, 2008a), central neural drive (Clark et al., 2006, 2008), H-reflex and spinal cord circuitry (Clark et al., 2006; Lundbye-Jensen and Nielsen, 2008b, 2008a), motor units firing rate (Sarto et al., 2022; Seki et al., 2001), EMG (Gondin et al., 2004; Sarto et al., 2022) and jitter (Grana et al., 1996; Sarto et al., 2022) are known to undergo significant changes. Consistent with these observations, in response to 4-week unilateral limb unloading in humans, it was concluded that 48% of the loss in force could be attributable to neural factors, 33% to muscular factors and 19% to other still unexplained causes (Clark et al., 2006).

The contribution of the alterations of the NMJ to the impact of neural factors on disuse-induced muscle force loss, in humans, is still unclear. However, several findings suggest denervation of muscle fibres and possible alterations of the NMJ following disuse.

Indeed, an increase in N-CAM positive fibres has been shown following even short periods of dry immersion (3d) (Demangel et al., 2017) and bed rest (BR) (5–10d), as well as of unilateral lower limb suspension (ULLS) (10d) (Arentson-Lantz et al., 2016; Monti et al., 2021; Sarto et al., 2022). An example of N-CAM positive fibres is shown in Fig. 2. Similarly, an increase in serum levels of CAF, a fragment of agrin, has been found after both 10 days of bed rest and 10 days of unilateral lower limb suspension in humans (Monti et al., 2021; Sarto et al., 2022). Associated with these changes were a decrease of SERCA 2 protein, involved in the reuptake of Ca^{2+} from the sarcoplasm to the sarcoplasmic reticulum, together with a reduction in Ca^{2+} release by the SR, suggesting impaired ECC following disuse, potentially contributing to the reduction in intrinsic force generation capacity with disuse (Monti et al., 2021).

Another sign of denervation is the expression of the sodium channel NaV1.5, which should be expressed concomitantly with N-CAM, however no data are reported on human models of disuse (Hendrickse et al., 2018). Furthermore, a time-course performed on animal model suggested that N-CAM and NaV1.5 may not be expressed at the same time and in response to denervation, supporting the importance of N-CAM positive fibres observed in humans following disuse (Demangel et al., 2017; Hendrickse et al., 2018; Monti et al., 2021; Sarto et al., 2022). In fact, N-CAM expression could be showing in some subjects just the tip of the iceberg (Hendrickse et al., 2018).

An NMJ involvement in muscle unloading has been highlighted in short as well as long periods of disuse in humans, and the molecular scenario appears still puzzling. For example, homer 2 was shown to decrease following 60 days of bed rest (Salanova et al., 2011). Such phenomenon is accompanied by a reduction of NFATc1 nuclear localization in slow muscle fibres, with an expected muscle protein synthesis decrease and/or fibre switching (Salanova et al., 2011). A reduced muscle protein synthesis may also depress protein level of important NMJ receptors, such as AChRs. However, not only protein level but also aggregation of AChRs may be affected by disuse.

In fact, AChRs aggregation is crucial to have a functional NMJ transmission. It also depends on the neurotransmitter nitric oxide (NO) that favours the agrin-mediated AChRs aggregation, acting as downstream mediator as shown in vitro (M. A. Jones and Werle, 2000). Interesting data about a NO role in NMJ integrity are reported in humans. NO is released by the neuronal nitric oxide synthase (nNOS or NOS1). Disappearance of nNOS from the sarcolemma, alongside with an increased expression of Gadd45a protein and N-CAM positive fibres, have been associated with human denervation in pathological contexts (Bongers et al., 2013; Ehmsen et al., 2021; Gosztonyi et al., 2001). The expression of these denervation-associated molecules is proved to be controlled by histone deacetylases 4 (HDAC4), at least for Gadd45a and only reported in animal model (Bongers et al., 2013; Moresi et al., 2010b). In human disuse, the decrease of active sarcolemmal nNOS has been correlated to increment of N-CAM suggesting its NMJ involvement (Arentson-Lantz et al., 2016; Demangel et al., 2017; Lechado i Terradas, A., Vitadello et al., 2018). In fact, the sarcolemmal nNOS is the active form of the enzyme at the NMJ side. During disuse nNOS relocation is also associated to the activation of FoxO3 pathway and atrogenes expression resulting in muscle protein degradation, but only evidence in HU rats are reported (Lechado i Terradas, A., Vitadello et al., 2018). However, a similar role could be most likely proposed in humans as suggested by the involvement of nNOS in AChRs aggregation during disuse, and maybe could be influencing NMJ homeostasis.

Although the evidence of NMJ instability in response to muscle

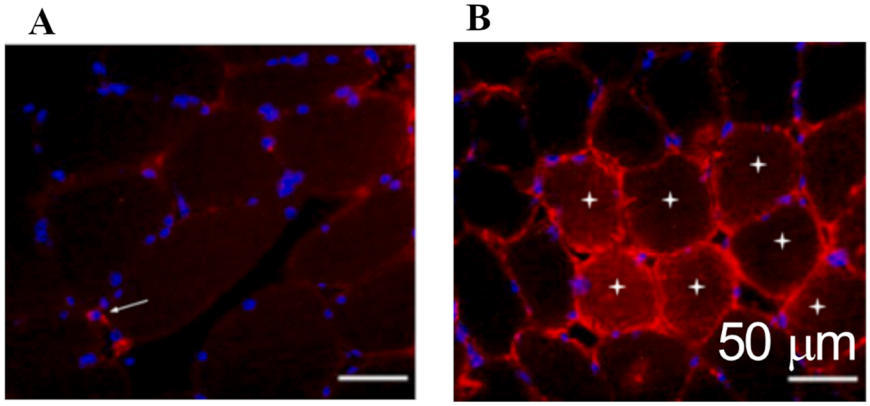

Fig. 2. Immuno-staining of N-CAM positive fibres on human vastus lateralis.

unloading is rather robust, the impact of these molecular and structural alterations on NMJ transmission properties is yet not clear. Indeed, despite evidence of changes in motor unit action potential properties and muscle fibre conduction velocity was found in response to short-term (10-day) bed rest and ULLS, no changes in NMJ transmission could be found in vivo after ULLS or limb immobilisation (Inns et al., 2022; Sarto et al., 2022). It is possible that this lack of transmission impairment is simply a temporal phenomenon and that it would manifest itself after longer period of disuse, beyond 15 days of muscle unloading. Future studies should investigate this hypothesis.

3.5. Emerging biomarkers of disuse: neurofilaments and axon damage

Disuse-mediated damages can also include motoneurons, which releases the axon components light or heavy neurofilaments externally. Interestingly, axons' damage has been associated with disuse atrophy in murine model (Banzrai et al., 2016). Neurofilaments are mainly considered in pathological contexts where consequently to extreme axon damage and neural death, the neurofilaments of axons are released into circulation. It was unknown if less severe conditions such as disuse, may also mediate a sort of axons' damage responsible of the release of neurofilaments into circulation. Interestingly, the serum detection of neurofilaments is emerging as potential biomarkers of axon damage at peripheral level, and has been associated to 10 days of ULLS in young males (Ivakine and Cohn, 2014; Salanova et al., 2014; Sarto et al., 2022). During disuse, the potential axon damage may exacerbate the alteration in neuromuscular transmission; as well as may be among the triggering mechanisms in favouring NMJ instability observed in the 10-day disuse window and reviewed here. An overview of the commented mechanisms is proposed in Fig. 3.

3.6. Release of specific neural factors in disuse

Neurotrophins are the molecules used to communicate between muscles and nerves (Nishimune et al., 2014). The alteration in the release or in the sensitivity (i.e., the expression level of their receptors TrkA, TrkB and TrkC) to neurotrophins may mediate NMJ instability. The plasma level of BDNF increases after bed rest only in aged population, but not in the young counterpart with no data available on other molecular players involved in NMJ stability (Ganse et al., 2021; Passaro et al., 2017; Soavi et al., 2016). No changes were reported also for GDNF in human bed rest (Ganse et al., 2021).

Evidence from disuse animal model show increased BDNF mRNA and protein level both in the spinal cord and soleus muscle after hindlimb unloading (Yang and Zhang, 2016), suggesting that neurotrophins are involved in neuromuscular plasticity following disuse. Interestingly, deletion and overexpression of BDNF in skeletal muscle affects motor end plate volume in conjunction with an effect on fibre-type transition (Delezie et al., 2019). BDNF overexpression increases fast-type gene expression and glycolytic fibres, the same muscle phenotypic transition associated with disuse. This suggests how BDNF could be the major player among the released neurotrophins that act on neuromuscular plasticity in response to disuse, especially in advanced age.

However, data on the level of these trophic factors in the different human disuse models and knowledge on their alleged protective role in NMJ homeostasis in conditions of disuse is scanty and incomplete.

The study of the NMJ morphology in human model of disuse is still lacking, even though new methods are emerging to detect NMJs-enriched site for biopsies (Aubertin-Leheudre et al., 2020). No data are available in humans either on lipid rafts disturbances or ceramides at the NMJ. Interestingly, circulating ceramides have been associated to cardiovascular risk and brain aging, also after short BR in humans emphasizing that further research are needed to better comprehend the human counterpart of disuse (McGrath et al., 2020; Petrocelli et al., 2020).

4. NMJ & aging: what can we learn from disuse?

Aging and disuse share common features (Fig. 4) among which oxidative stress, inflammation, mitochondrial alterations in term of dynamics and biogenesis, and an altered muscular metabolism (Hood et al., 2019). The study of molecular pathways acting during disuse could help in unravelling important players of aging, a condition in which such molecular and physiological alterations may have a similar cause but with chronic implications. NMJ fragmentation was observed not only in disuse, but also in aging rodents (Chai et al., 2011; Valdez et al., 2010), even though some studies reported that it could not be a valid indicator of alteration of neuromuscular transmission (Slater, 2020; Willadt et al., 2016). It should be noted that the study by Willadt et al., was conducted on diaphragm muscle, which is chronically active, and not on a typical muscle involved in voluntary movements (Willadt et al., 2016). Furthermore, Slater et al., in their review commented

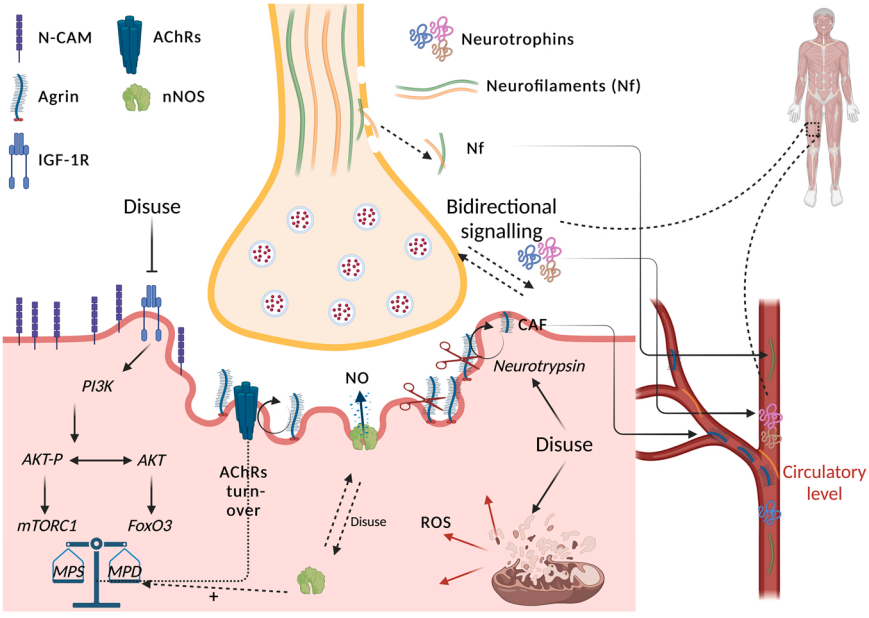

Fig. 3. Overview of the effects of disuse on NMJ related pathways.
An overview of the evidence reported in humans and in animal models and related to the effects of disuse on the NMJ, are presented in the figure. One of the initial molecular events is the insulin-like growth factor 1 (IGF-1) axis inhibition, with related increase in muscle protein degradation (MPD). MPD and autophagy also affects acetylcholine receptors (AChRs) turn-over. Even AChRs aggregation is repressed due to the augmented cleavage of agrin by neurotrypsin, with consequent release of c-terminal agrin fragment (CAF) into the blood. Disuse induces reactive oxygen species (ROS) release by mitochondria, with sarcoplasm re-localization of the neuronal nitric oxide synthase (nNOS) that favors MPD in an unknown way. N-CAM de-novo expression and its sarcolemma localization are among the events induced by disuse-denervation events. The bidirectional signalling and exchange of neurotrophins between muscle and nerves may be affected by disuse, in ways to be clarified. During disuse light neurofilaments are released most likely due to peripheral axon damages.

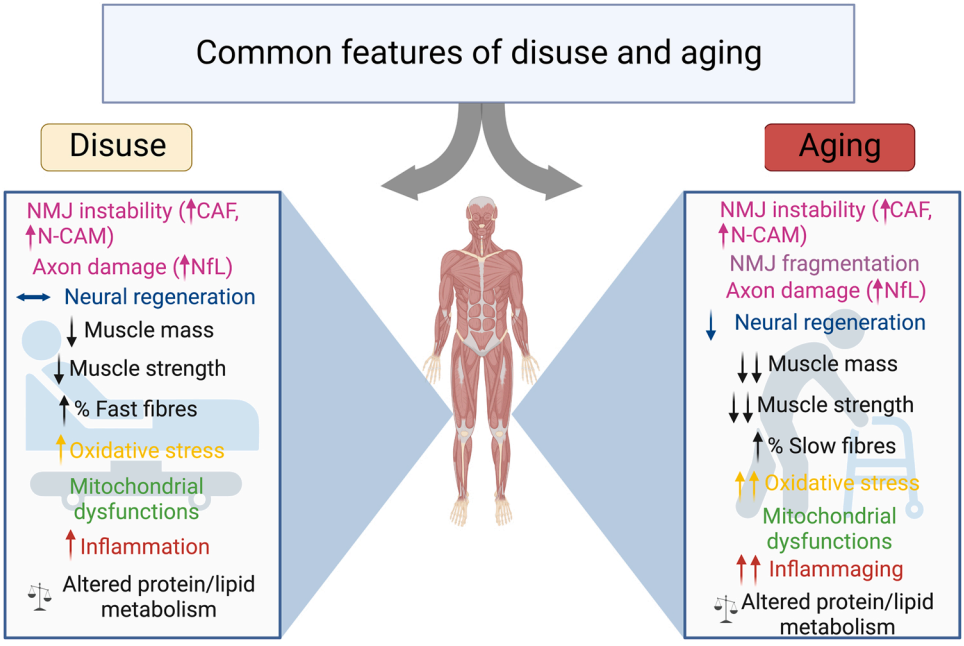

Fig. 4. Overview of the common features between disuse and aging.
Disuse and aging share common features, even though at different rate and with molecular and physiological contributions to be clarified. NMJ instability and axon damage were observed in disuse and aging, with common biomarkers (CAF, N-CAM and NfL). NMJ fragmentation seems to be peculiar of aging, even though more studies are needed. The capacity of motoneurons to re-innervate skeletal muscle fibres is maintained after disuse, but it is progressively less efficient during aging, as suggested by the response to the physical activity. Muscle mass and strength are lost in both conditions, but more pronounced in aging of which sarcopenia is the extreme. Muscle fibres population shows more fast fibres with disuse, and more slow fibres with aging. The latter could be due to fibre switching, as well as to reduced fast fibres percentage. Mitochondrial dysfunctions and inflammation occur in both conditions, even though a peculiar chronic inflammation, called inflammaging, is typical of aging. Finally, an altered protein-lipid metabolism contributes to the unfunctional changes.

about NMJ fragmentation as an occurrence of regeneration and not degeneration (Slater, 2020). The issue remains partially unsolved since regeneration would imply that previous degeneration occurred. Further studies should investigate whether these phenomena can be part of a cycle.

Moreover, humans could behave differently: in fact, the NMJ instability observed in older individuals, seems to be related to the level of physical activity of the subjects (Franchi et al., 2022; Marcolin et al., 2021; Pratt et al., 2021, 2022). Further research should clarify the contribution of the disuse-related molecular players to muscle aging, such as agrin, neurofilaments, hemichannels and N-CAM.

The molecular mechanisms discussed here may open new perspectives on the aging research, highlighting the need to establish which are the common players between disuse and aging in NMJ instability, and how elders respond to disuse compared to younger adults. In turn, this could help to establish appropriate countermeasures to combat both aging-related and disuse-related alterations in clinical practice.

5. Conclusions

The NMJ is the key site for electrical stimuli transmission enabling muscle contraction, with retrograde signals such as circulating neurotrophins and myokines able to 'feed' and drive motoneuron transcriptional program. The bidirectional signal is fundamental for muscle homeostasis as well for neurons. Maintenance of a constant electrical activity, disrupted by sedentarism and chronic inactivity, seems to be the key factor for preserving neuromuscular system.

Studies of the NMJ adaptations to disuse are few especially in humans. In mice, several findings indicate NMJ morphological and molecular adaptations. Interestingly, they can occur within hours of unloading and might trigger not only muscle mass loss, but also other key phenomena of disuse such as altered mitochondrial dynamics and insulin resistance. In humans, given the persisting difficulties in studying the morphology of the NMJ, evidence is mostly indirect being based on markers of denervation such as N-CAM expression and circulating CAF. Such markers indicate signs of the NMJ instability after just 3–10 days of muscle unloading.

Importantly, findings so far surely indicate a great plasticity of the neuromuscular system in response to disuse and potential detrimental adaptations. The field of the NMJ remodelling in disuse is promising, but a lot of work is still needed to clarify the whole picture.

CRediT authorship contribution statement

Conceptualization: G.S.; data curation: G.S.; Formal analysis: G.S.; Funding acquisition: M.V.N.; Supervision: M.V.N.; Roles/Writing - original draft: G.S.; Writing - review & editing: G.S., M.A.P., R.B., M.F., M.V.N.

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Conflict of interest

The authors declare no conflict of interest.

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