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REGULATION OF MUSCLE SATELLITE CELL
PROLIFERATION AND DIFFERENTIATION
BY LOCAL TROPHIC FACTORS

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The skeletal muscle is a terminally differentiated tissue. Its capacity to repair following injury or disease depends on a population of myogenic precursors, named satellite cells. These cells are localized beneath the skeletal muscle fiber, in a specialized microenvironment, the niche. The niche preserves the homeostatic conditions of satellite cell quiescence, but at the same time, it ensures their responsiveness to mechanical, physical and chemical triggers from the surrounding environment. Therefore, the composition of the external milieu is critical in determining satellite cell behavior. As a matter of fact, during aging or under pathological conditions, alterations of the extracellular environment entail a severe impairment of satellite cell ability to sustain regeneration and repair of the skeletal muscle tissue.

The general goal of this thesis was to focus on some of the trophic factors potentially present in the satellite cell niche in vivo and to characterize their role on the modulation of satellite cell functions in vitro.

The first part of the research activity dealt with the study of the trophic effect of ATP on mouse myoblast proliferation. From literature, it emerged that ATP is a potential regulator of the skeletal muscle regenerative program, however the signalling mechanism remained partially unknown. We observed that ATP increased myoblast growth rate, effect that was mimicked by low concentrations of H₂O₂. Reactive oxygen species (ROS) imaging revealed that ATP induced H₂O₂ production, at concentrations comparable to those effective in triggering myoblast proliferation. Interestingly, the exposure to equimolar concentrations of adenosine did not mimic the effect of ATP, excluding any role for the main hydrolysis product of ATP in the control of cell cycling. This result was in agreement with data reporting that the specific enzymes responsible for ATP degradation are poorly expressed in myoblasts and become upregulated after cell differentiation.

In line with the latter observation, it appeared reasonable that the differentiating skeletal muscle cells were more exposed to ATP-derived adenosine than proliferating myoblasts, and this suggested a potential physiological role for the nucleoside adenosine in the later phases of myogenesis. Taking into account that adenosine receptors (ARs) are present in mouse myotubes, in a second study we hypothesized a crosstalk between nAChRs and ARs. Using the Ca²⁺-imaging technique, we observed that the pharmacological modulation of ARs triggered variations in the nAChR-driven ([Ca²⁺]ᵢ) spikes. Moreover, our preliminary
results suggest not only an interplay between the two receptors but also that endogenous adenosine is tonically released by twitching myotubes and activates its receptors.

The third research project was aimed at exploring the role of neural agrin, a heparan sulphate proteoglycan, so far known as the key organizer of post-synaptic elements during skeletal muscle differentiation/regeneration. Besides agrin's canonical effect on the maturation of the NMJ, novel roles have been discovered in the recent years, suggesting that the neurotrophic factor has pleiotropic effects. In this new context, we pursued the identification of potential new roles for neural agrin in the determination of satellite cell behaviour. Firstly, the analysis of different cell models, including C2C12 cell line and primary mouse and human cells, and revealed an increase in IL-6 secretion following exposure to agrin. Secondly, we addressed the hypothesis of agrin as a potential modulator of human myoblasts proliferation. Our preliminary results demonstrate that agrin enhances the proliferative capacity of human satellite cells and suggest the potential mechanism involved in the signaling cascade.
Il muscolo scheletrico è un tessuto terminalmente differenziato. La sua capacità rigenerativa in seguito a danno o patologia dipende da una popolazione di precursori miogenici, le cellule satellite. Esse sono localizzate sulla superficie della fibra muscolare, racchiuse in un ambiente altamente specializzato, la nicchia. La nicchia assicura il mantenimento della quiescenza cellulare, ma allo stesso tempo fa sì che la cellula satellite risponda a stimoli meccanici, fisici o chimici, provenienti dall’ambiente esterno. Per questo motivo, la composizione dell’ambiente circostante condiziona altamente il comportamento della cellula satellite. Infatti, le alterazioni che si verificano con l’invecchiamento o in seguito a patologia compromettono la capacità dei precursori miogenici di sostenere la rigenerazione del tessuto.

Lo scopo di questo lavoro di tesi è stato quello di individuare e caratterizzare alcuni dei fattori trofici che compongono il microambiente della cellula satellite in vivo, per cercare di capire come essi modulino le funzioni dei precursori miogenici durante la rigenerazione in vitro.

La prima parte dell’attività di ricerca ha riguardato lo studio dell’effetto trofico dell’ATP sulla proliferazione mioblastica. Studi in letteratura hanno fatto emergere il potenziale ruolo regolatore dell’ATP nella rigenerazione muscolare, anche se i meccanismi attraverso cui opera non sono ancora chiari. I risultati da noi ottenuti hanno dimostrato che l’ATP aumenta la proliferazione mioblastica e che un effetto simile si osserva in presenza di H₂O₂. L’imaging per le specie reattive dell’ossigeno (ROS) ha inoltre dimostrato che l’ATP induce la produzione di H₂O₂, a concentrazioni paragonabili a quelle capaci di aumentare la proliferazione.

In presenza di concentrazioni equimolari di adenosina non è stato osservato alcun effetto sulla proliferazione cellulare, fatto che suggerisce che il ruolo dell’ATP non sia attribuibile all’adenosina, il suo principale prodotto di degradazione. Questo risultato è in accordo con quanto già riportato da altri Autori a proposito degli enzimi responsabili dell’idrolisi dell’ATP: essi sono poco espressi nei mioblasti in proliferazione, mentre la loro espressione aumenta notevolmente con il differenziamento cellulare.

Alla luce di queste osservazioni, risultava verosimile che i miotubi fossero più esposti rispetto ai mioblasti all’adenosina derivante dall’ATP e che pertanto l’adenosina avesse un ruolo fisiologico preponderante nelle fasi avanzate della miogenesi. Dal momento che i
recettori per l’adenosina (ARs) sono espressi nei miotubi murini, un secondo lavoro ha avuto come obiettivo quello di investigare un possibile “crosstalk” tra i ARs e i nAChRs. Esperimenti di Ca\(^{2+}\)-imaging hanno dimostrato come la modulazione farmacologica dei ARs si traduca in una variazione nelle oscillazioni di [Ca\(^{2+}\)], indotte dall’attività del nAChR. Questi risultati, sebbene preliminari, suggeriscono non solo che i due recettori interagiscano tra loro, ma anche che l’adenosina è tonicamente secreta dai miotubi in contrazione e agisca attivando i suoi recettori.

Il terzo progetto di ricerca è stato finalizzato allo studio del ruolo dell’agrina neuronale, un proteoglicano eparan solfato, già noto per la sua capacità di aggregare elementi sinaptici durante la fase di differenziamento e di rigenerazione del muscolo scheletrico. Accanto al ruolo canonico che la vede coinvolta nella maturazione della giunzione neuromuscolare, negli ultimi anni sono state descritte nuove funzioni per l’agrina neuronale, che la indicano come un fattore pleiotropico. In questo contesto, abbiamo esplorato nuove proprietà dell’agrina. In primo luogo, l’analisi di diversi modelli cellulari, incluse la linea cellulare C2C12 e cellule primarie murine e umane, ha dimostrato che il fattore neurotrofico potenzia il rilascio di IL-6. In un secondo studio, è stato ipotizzato un potenziale effetto di modulazione della proliferazione di mioblasti umani da parte dell’agrina neuronale. Risultati preliminari hanno dimostrato che l’agrina aumenta la capacità proliferativa delle cellule satelliti umane. Inoltre, sono stati individuati alcuni dei fattori molecolari che partecipano alla cascata di segnalazione.
1. The satellite cell: discovery and anatomical distribution

Adult skeletal muscle is composed of highly aligned, multinucleated and cross-striated fibers that are terminally differentiated. Tissue homeostasis and repair depend on a population of muscle specific stem cells, named satellite cells. Satellite cells were discovered more than 50 years ago by Mauro, who noticed this population of mononucleated cells on the skeletal myofiber surface\(^1\). When these cells were discovered, it was noticed that they were distributed in a peculiar manner like a “satellite” at the periphery of the myofiber, thus giving rise to their name. Their specific localization on the skeletal muscle fiber surface suggested Mauro these cells to be undifferentiated precursors, capable of forming new tissue after injury. After their identification, when the physiological significance of these cells was still not clear, there were two hypotheses about their mechanism of action. It was first stated that activated precursors absorb the cytoplasm of the underlying damaged myofiber and gain its functional properties before substituting it. The second hypothesis considered satellite cells as specific inactivated precursors of the skeletal muscle fiber, remaining in a quiescent state until activation.

To date, it is known that satellite cells reside in indentations between the sarcolemma and the basal lamina, in a specialized microenvironment called niche (Fig. 1)\(^1,2\). The niche houses these progenitor cells in their quiescent state but, at the same time, prepares them to be readily responsive to molecular triggers induced by physical exercise, injuries or disease. Furthermore, it is recognized their essential and active participation in tissue regeneration and their preponderant involvement in comparison to other myogenic skeletal muscle precursors. This is why satellite cells are considered a challenging opportunity in the emerging field of the regenerative medicine, and in providing new therapeutic strategies to cure skeletal muscle pathologies and diseases.

Next sections will provide an overview about the process of skeletal muscle regeneration, how satellite cell are activated and the complex network of stimuli belonging to the extracellular environment that controls satellite cell behavior.
Fig. 1. The satellite cell is located on the skeletal muscle fiber surface and under basal physiological conditions is enclosed in the niche, composed by the basal lamina (apical surface) and plasma membrane (basal surface) of the underneath skeletal muscle cell (from [3]).

2. The skeletal muscle regeneration

The skeletal muscle regeneration consists of 4 time-dependent phases, highly interconnected to each other: degeneration, inflammation, regeneration and remodeling/repair. The successful accomplishment of each of these steps is required to fulfill the subsequent phase, allowing an efficient tissue regeneration process.

2.1. Degeneration

The first phase of skeletal muscle regeneration is characterized by necrosis of the myofiber after injury. It is due to the influx of high levels of extracellular calcium, inducing proteolysis of the myofibers. Although the necrotic process destroys and damages the tissue, it induces the activation of the inflammatory response and represents the first step towards the neo-formation of the tissue.

2.2. Inflammation

In this phase, inflammatory cells are recruited to remove the necrotic debris. Neutrophils are the first cells to invade the damaged site, where they rapidly release high
concentrations of free radicals and proteases. The oxidative and proteolytic modifications carried out by neutrophils promote the clearance of cellular debris by macrophages, which will subsequently arrive and act as new players. There are two types of macrophages involved in a time-dependent manner: at the beginning, M1 infiltration leads to the release of pro-inflammatory cytokines and of potentially harmful oxygen-free radicals, that can damage the tissue. Later, M2 macrophages are activated. They are anti-inflammatory cells releasing low amounts of pro-inflammatory cytokines. The action of macrophages and neutrophils may lead to severe muscle damage due to the release of very reactive molecules. However, the role of inflammatory cells is essential for successful tissue turnover. In fact, alterations in the inflammatory response induced by drugs, aging or pathological conditions cause a severe impairment of muscle regeneration.

2.3. **Regeneration**

The extensive release of growth factors and cytokines during the inflammatory phase induces the activation of satellite cells. The undifferentiated progenitors break cellular quiescence and enter the cell cycle and proliferate; later on, they stop cycling, fuse together and differentiate. The result is the formation of new functional myofibers. The activation of satellite cells depends on several growth factors released at the injured site, including insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factors (TGFα and TGFβ), platelet-derived growth factors (PDGF-AA and PDGF-BB).

2.4. **Remodeling/repair**

The final remodeling/repair phase is characterized by the functional recovery of the injured skeletal muscle, together with angiogenesis and connective tissue restoration. The remodeling of the extracellular matrix (ECM) has a great impact on the final outcome of the regenerative process. In fact, muscle injury generates an alteration of the ECM components, which ultimately induces the production of several types of collagens to accomplish remodeling. The efficient recovery of muscle functions requires a perfect balance between fiber and scar tissue formation.
3. Satellite cells and skeletal muscle regeneration

As mentioned above, under basal conditions satellite cells are kept in a quiescent state, so that they are transcriptionally low active, with minimized oxidative stress and preserved mitotic potential. In fact, they have both a high amount of nuclear heterochromatin and nuclear to cytoplasmic ratio, few organelles and a smaller nuclear size, as compared to that of the adjacent myofiber\(^2\) (Fig. 2). Moreover, they exhibit limited gene expression and protein synthesis. After their activation, satellite cells become mitotically active, and from a morphological point of view, they are more easily identifiable. They display cytoplasmic processes to allow the migration along the myofiber, own a higher number of intracellular organelles, a lower amount of heterochromatin and an increased cytoplasmic to nuclear ratio\(^2\) (Fig. 2).

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**Fig. 2.** Under normal physiological conditions the satellite cell is maintained in its sublaminar position in a quiescent state, displaying a high nuclear to cytoplasmic ratio, and a smaller nuclear size, as compared to the one of the myofiber. Upon injury of the muscle fiber, the satellite cell becomes activated, increases its cytoplasmic content and displays cytoplasmic processes (from \(^{[11]}\)).
Activated satellite cells migrate outside the niche and start cycling. The descendants of activated satellite cells, called myoblasts, undergo multiple rounds of divisions in order to expand the population, and express myogenic markers. In a further phase, myoblasts stop cell cycle, aligned themselves and fuse together. The result is the generation of multinucleated cells, called myotubes, the precursors of skeletal muscle fibers. A small number of satellite cells doesn’t undergo the myogenic commitment\textsuperscript{12}, being the fraction of progenitors that will replenish the satellite cell pool in the depleted niches\textsuperscript{13}.

3.1. **Molecular characterization of satellite cells during quiescence, proliferation and differentiation**

Satellite cell myogenic potential relies on the expression of the paired-box transcription factors 3 and 7 (Pax3, Pax7) and myogenic regulatory factors (MRFs). Pax3 and Pax7 are important regulators of embryonic and adult myogenesis. Although their amino acidic sequences are very conserved, they show overlapping roles only for the myogenic specification.

Pax3 function is still not well defined. During the embryonic development, Pax3 is critical for the delamination and migration of muscle progenitor cells from the somites to the limb buds\textsuperscript{14}. In the post-natal period, Pax3 is progressively downregulated and remains detectable only in few muscle groups, such as the diaphragm and the body wall\textsuperscript{15}. The fact that most muscles downregulate it after birth suggests that it has a minor role in postnatal myogenesis, although this issue remains still controversial. Pax7 has a more crucial function during postnatal growth with respect to Pax3 and is required for the myogenic specification of satellite cells\textsuperscript{16}. Its expression correlates with an undifferentiated but committed myogenic state during the proliferative phase\textsuperscript{17} (Fig. 3), and is required for maintenance, specification and development of satellite cells\textsuperscript{18}. As demonstrated by knockout experiments in mice, the lack of Pax7 expression leads to satellite cell lost, reduction in muscle mass and defective regenerative capacity\textsuperscript{19,20}. As soon as myoblasts approach the myogenic commitment, Pax7 is rapidly downregulated and other myogenic markers are activated\textsuperscript{12}. Among these markers, it is noteworthy to mention the myogenic factor 5 (MYF5), and the myoblast determination protein (MYOD), which starts to be expressed during the proliferative phase\textsuperscript{21} (Fig. 3).
The transition of myoblasts into myocytes and myotubes is regulated by myogenin, a downstream target of MyoD, and MRF4 (Fig. 3). Myogenin and MRF4 are not required for satellite cell maintenance and activation, but their lack may compromise cell differentiation and myofiber formation. In particular, the inactivation of the myogenin gene leads to the accumulation of undifferentiated myoblasts.22

**Fig. 3.** Myogenesis is a strictly orchestrated process in which activated satellite cells, called myoblasts, proliferate and subsequently differentiate to provide new functional myofibers. Each phase is tightly regulated by a specific gene expression profile that determines satellite cell fate (self renewal; from [21]).

### 3.2. Satellite cell self-renewal

The ability of self-renewal is a hallmark of stem cells and is the mechanism through which a copy of the parental stem cell is preserved in order to maintain a constant number of progenitors in a certain tissue. In the skeletal muscle, the self-renewal capability of activated satellite cells is an important mechanism that preserves muscle regenerative ability throughout adult life.

Although, in the past, satellite cells have long been considered a homogeneous population of committed progenitors, the ability of satellite cells to self-renew indicates that they belong to a heterogeneous population of stem-like and committed cells.24 In fact, the total number of quiescent satellite cells remains constant during multiple rounds of regeneration, and the balance between committed and uncommitted satellite cells is a peculiar feature of skeletal muscle regeneration.13 This is further proven by the fact that
not all progenitors display the same mitotic activity. It was shown that 80% of satellite cells are fast-cycling, whether the last 20% represents a fraction of slow-cycling reservoir\textsuperscript{25}. While the first are actively participating to the regenerative process, the latter preserve their stem-like properties. These distinct fates were observed even in transplantation experiments. In fact, engrafted cells were both able to be activated to provide new functional differentiated myofibers and to replenish the reservoir pool\textsuperscript{13,26}. Satellite cell division may occur in a planar or in an apical-basal orientation with respect to the basal lamina (apical surface) and to the sarcolemma of the host fiber (basal surface). The orientation of cell division depends on specific elements present in the niche and determines the fate of the daughter cells, depending on their position within the niche. In the planar division, there is no polarization of the niche and both cells remain in contact with the basal lamina and with the myofibre; in the apical-basal division, a daughter cell is pushed towards the basal lamina and the other towards the myofibre (Fig. 4). Kuang and collaborators observed that in the basal division, the maintenance of the same position for the doublet of cells gives rise to two progenitors with the same fate (symmetric division)\textsuperscript{27}. In the apical-basal division, the loss of the contact of one daughter with the basal lamina and the extracellular matrix leads to the generation of progeny with two distinct fates (asymmetric division). The hypothesis of the immortal strand postulates that during the asymmetrical cell division the segregation of DNA chromatids occurs non randomly. For each division, the DNA template of the parental cell is destined to only one of the two daughters, which is the self-renewing stem cell. Thus at every cycle, a copy of the “old” inactivated progenitor is preserved. The advantage of the asymmetric division is to ensure the recovery of the original stem-cell phenotype of the parental progenitor and to minimize the accumulation of mutations related to cell replication\textsuperscript{28}. Unlike other stem cells, satellite cells can undergo both asymmetric and symmetric division; the choice between one or another depends on the state of injury or disease triggering satellite cell activation.

The heterogeneous population of satellite cells can be classified depending on MYF5 expression. Committed satellite cells are PAX7\textsuperscript{+}/MYF5\textsuperscript{+} while non committed progenitors are PAX7\textsuperscript{+}/MYF5\textsuperscript{-}. Strikingly, it was observed that planary dividing cells are either PAX7\textsuperscript{+}/MYF5\textsuperscript{-} or PAX7\textsuperscript{+}/MYF5\textsuperscript{+}, while cells dividing with an apical-basal orientation are predominantly PAX7\textsuperscript{+}/MYF5\textsuperscript{-} and PAX7\textsuperscript{+}/MYF5\textsuperscript{+} in the basal and apical position,
respectively. Committed PAX7+/MYF5+ daughters will be destined for terminal differentiation; Pax7+/Myf5− cells will replenish the stem cell-like reservoir (Fig. 4).

![Schematic representation of satellite cell fate in symmetric and asymmetric division.](image)

**Fig. 4.** Schematic representation of satellite cell fate in symmetric and asymmetric division. Quiescent satellite cells are PAX7+/MYF5−. After activation, the apical-planar division distinguishes the genotype of the daughter cells (PAX7+/MYF5+ vs. PAX7+/MYF5−). Viceversa, the planar orientation of cell division gives rise to daughter cells with symmetric PAX7 and MYF5 expression (from [29]).

### 4. The critical role of the satellite cell niche

The niche is a highly specialized microenvironment profoundly affecting satellite cell behavior and regulating their contribution to tissue regeneration, maintenance and repair. As already mentioned, this specific anatomic location is composed in its apical part by the basal lamina, which is in direct contact with components of the ECM, and in its basal surface by the sarcolemma of the associated skeletal muscle fiber. This microenvironment transmits biochemical and structural cues to the satellite cell and integrates signals from the external environment. Moreover, the niche saves satellite cells
from depletion, preventing excessive activation and ensuring the maintenance of a stem cell pool during regeneration.

To better figure out the composition of the niche, we can imagine it to be inserted into a three-layered system. The so called “immediate cell niche” is constituted by the satellite cell itself, the myofiber and the extracellular matrix. The immediate niche is surrounded in turn by the so called “local milieu”, conditioned from all the neighboring cells, such as motor neurons, adipocytes, fibroblasts, capillaries, endothelial cells, connective tissue. Finally, the local milieu interacts with a broader environment, the “systemic milieu”. The whole integrated system provides a plethora of molecular signals, creating the instructive environment essential for satellite cell quiescence, as well as for proliferation, self-renewal, commitment and differentiation during skeletal muscle regeneration\textsuperscript{30}.

The main anatomical and molecular features of the niche are described hereafter.

4.1. The “immediate niche”

As mentioned above, the “immediate niche” refers strictly to the microenvironment where the satellite cell is placed. The satellite cell sits on the myofiber with whom it communicates mechanically, physically and chemically. Due to this close relationship, the satellite cell is very susceptible to the mechanical activity of the underlying myofiber and, under specific conditions, the contractile stimulus is transduced by the satellite cells. In addition, physical interactions are allowed by cell adhesion proteins, which create an intimate contact with the myofiber. The extracellular matrix (ECM) as well is physically associated with the satellite cells, and its stiffness and composition contribute to the regulation of their behaviour. Last but not the least, the myofiber releases, often in an activity-dependent manner, soluble factors such as IGF, HGF, bFGF, Wnt7a, to whom satellite cells are exposed (Fig. 5).

4.1.1. Mechanical stimuli

In normal conditions, basal muscle contractions and relaxations occur and resident satellite cells are kept in a quiescent state. When muscle is injured, exercised, overused or mechanically stretched, these cells become activated and start the regenerative program. Therefore, specific mechanical changes are perceived by the myogenic precursors. For
example, it is well known for example that mechanical contraction of the myofiber triggers the production and release of nitric oxide (NO). NO affects the autocrine production of an important growth factor, the hepatocyte growth factor (HGF), that is a crucial stimulator of satellite cell activation\textsuperscript{32}.

\textbf{Fig. 5.} The figure shows a representation of the “immediate niche”, regulating satellite cell function and behavior. The satellite cell interacts physically and chemically with the underlying skeletal muscle fiber and with components of the ECM (modified from \textsuperscript{31}).

\subsection{Cell-cell interactions}

Cell-cell interactions represent an important regulatory mechanism for the satellite cell fate. Satellite cells express cell adhesion molecules such as neural cell adhesion molecule (NCam), cadherins, and vascular cell adhesion protein (VCam).

Neuronal cell adhesion molecules (NCam) are integral membrane glycoproteins, mainly studied to mediate interneural interactions in the peripheral and central nervous systems. NCam are also expressed in the skeletal muscle, where their function is to communicate to the nerve the status of fiber innervation. In fact, in embryonic and denervated muscle NCam levels are very high whereas, in adult and reinnervated muscles, NCam expression is reduced\textsuperscript{33}. Immunostaining of adult skeletal muscle tissue revealed that these glycoproteins are concentrated at the neuromuscular junction (NMJ)\textsuperscript{34}. Furthermore, NCam are expressed occasionally also by satellite cells and during tissue regeneration by
myoblasts and myotubes\textsuperscript{34}, suggesting that they are also mediators of surface interactions of developing skeletal muscle precursors with nerve endings.

Cadherins belong to the family of Ca\textsuperscript{2+}-dependent cell adhesion molecules (Cam). Several subtypes of Cadherin have been identified, each one with its own pattern of distribution. Among them, the muscle isoform (MCad) is expressed by myogenic precursors\textsuperscript{35} and is located on the myofiber side. This pattern of expression suggests a role for MCad in stabilizing the contact between the satellite cell and the myofiber. Interestingly, during the regenerative program, MCad expression is upregulated and it seems to have a role in myoblast cell-cell interactions and recognition prior to fusion\textsuperscript{36}.

4.1.3. \textit{Interconnection with the components of the ECM}

The basal lamina is composed of proteins of the extracellular matrix (ECM) that are mainly synthesized and secreted by interstitial fibroblasts\textsuperscript{37}. The ECM molecules present in the basal lamina of the skeletal muscle fiber are collagen IV, laminin, entactin, fibronectin, perlecan, decorin glycoproteins, as well as other proteoglycans\textsuperscript{38}. These ECM molecules are directly linked to the cytoskeleton of the satellite cell. Their binding occurs via α7/β1-integrins, which are expressed on the satellite cell membrane and anchored to the actin cytoskeleton\textsuperscript{39}. These ECM components are thus crucial factors able to transmit physical interactions to the satellite cell, which in turn converts these signals into intracellular chemical ones\textsuperscript{40}.

Upon satellite cell activation, the ECM microenvironment undergoes a structural remodeling. In fact, the myogenic precursors have to cross the ECM barrier and abandon their niche to be available for tissue regeneration. The remodeling seems to occur through the secretion of matrix metalloproteinases (MMPs)\textsuperscript{41}. MMPs belong to a family of zinc-dependent enzymes, and while their expression is usually inhibited in quiescent satellite cells through specific protease inhibitors\textsuperscript{42}, they are upregulated during muscle injury\textsuperscript{41}.
4.1.4. *Chemical stimuli*

Myofibers and/or activated satellite cells synthesize and release molecules that act as trophic factors during muscle regeneration. Belong to the immediate niche: the hepatocyte growth factor (HGF), the fibroblast growth factor (FGF), the insulin-like growth factor (IGF-1) and proteoglycans.

The HGF is released by injured myofibers and is a satellite cell activator. In more detail, HGF plays a critical role in the earlier stages of myogenesis, stimulating satellite cell entry into the cell cycle and migration\(^43\). HGF effects are mediated by c-met receptor, which is present on skeletal muscle cells under physiological conditions, as well as on satellite cells\(^44,45\).

The FGFs are polypeptides that build up a family of over 20 members of mitogens. These soluble molecules, released by myofibers and by satellite cells, contribute to tissue homeostasis by favoring repair and response to injury\(^46,47\). The FGF family consists of several isoforms that exert differential and, in some cases, still controversial roles during myogenesis. FGFs signaling is mediated by four distinct transmembrane tyrosine kinase receptors (FGFR1-4), each with a different affinity for the members of the FGF family\(^48\).

Skeletal muscle and myogenic precursors express seven isoforms of FGF (FGF1-7). In particular, FGF2 and FGF6 are those playing a major role in the myogenic program\(^49,50\). Acting in an autocrine and/or paracrine fashion, they increase satellite cell activation and stimulate migration and differentiation\(^49\). The pleiotropic effect of FGFs in the skeletal muscle may derive from a double signaling cascade they can act through, depending of the expression of the receptor FGFR1 or FGFR4. Stable overexpression of FGFR1 correlates with satellite cell proliferation and delay of differentiation. On the contrary, FGFR4 expression starts when myoblasts become committed to fuse and differentiate.

Furthermore, it was shown that FGF6 is capable of inducing dedifferentiation of activated myogenic precursors. These observations suggest that FGF6, besides its contribution to skeletal muscle regeneration, owns an intrinsic mechanism to preserve a myogenic precursor pool from commitment, by dedifferentiation of activated myoblasts\(^50\).

The IGF-1 gene codes for multiple isoforms of IGF-1, which are the result of transcriptional and splicing variants, post-transcriptional regulation and post-translational modifications. All these processes contribute to the production of many peptides with
distinct physiological roles and patterns of distribution. IGF-1 is expressed in various tissues, such as liver and adipose tissue, where it is mainly released as a circulating peptide acting in an endocrine fashion. A specific IGF-1 isoform, called “muscle-IGF-1” (m-IGF-1) is expressed and produced by myofibers in response to injury or mechanical overload\(^{51,52}\). IGF-1 immunoreactivity was detected also in myoblasts and in myotubes\(^{53}\). Among all locally released growth factors, the attention in the few past years has particularly been focused on IGF-1 because of its unique pleitropic role both in proliferation and in differentiation of myogenic precursors. IGF-1 effect is mediated by its receptor IGF1R that triggers the activation of different signaling pathways: one drives to the phosphorylation of ERK, which is required for satellite cell proliferation\(^{52}\); the other is the AKT pathway, which drives to myoblasts differentiation\(^{54}\). It is also important to underline that the effects of IGF-1 depend on the origin of the molecule and that the autocrine/paracrine vs systemic mechanism of action of IGF-1 are independent. In fact, while the systemic infusion of IGF-1 does not affect muscle mass, the local administration of the growth factor is associated with muscle hypertrophy, selectively affecting only the muscle in which it was injected\(^{55}\). \textit{In vitro} superfusion of IGF-1 to C2C12 cells induces a marked increase in cell proliferation in a dose-dependent manner\(^{52}\). Local expression of IGF-1 modulates the inflammatory response, favoring tissue remodeling\(^8\).

Proteoglycans are located on the surface of satellite cells and they play a role in signal transduction in such a way that they regulate growth factor binding to their receptors. In particular, proteoglycans like syndecan3 and 4 sequester inactive growth factors, such as HGF, EGF, and FGF, IGF-1 and create a local reservoir to facilitate a rapid response of satellite cells to muscle injury\(^{56}\). Moreover, x-ray crystallography evidence indicates that HSGPs establish a double interaction with FGF receptor and are responsible for its dimerization and promotion of high affinity FGF binding\(^{57}\).

4.2. The “local milieu”

Unlike the immediate niche, the local milieu is made up of heterogeneous cell types, including interstitial cells, motor neurons and vascular cells (Fig. 6).
Fig. 6. The local milieu of the satellite cell niche is defined by all the components of the muscle fascicle. It is characterized by high cell type heterogeneity and it is made up of myofibers, interstitial cells, like fibroblasts or adipocytes, motor neurons and capillaries (modified from [30]).

4.2.1. Interstitial cells

Fibroblasts are the main components of the interstitial space, which is located between the basal lamina and the epimysial sheet surrounding the skeletal muscle. There is a well-regulated homeostasis between fibroblast activity and satellite cell behavior. It was demonstrated that in vitro co-culturing of fibroblasts and myoblasts ameliorates and promotes the commitment to terminal muscle cell differentiation\(^{58}\). In the light of this observation, it has been proposed that fibroblasts actively participate to the microenvironment of satellite cells and contribute to muscle regeneration through the secretion of growth factors, such as FGF, and of ECM molecules\(^{59}\) (Fig. 6).

4.2.2. Motor neurons

The status of muscle innervation directly influences satellite cell behavior and regenerative ability. Besides the neuron-derived myofiber electrical stimulation, generating physical and chemical signals in the satellite cell, motor neurons (Fig. 6) also secrete soluble trophic factors, such as the nerve trophic factor (NTF) and the brain-derived neurotrophic factor (BDNF). Moreover, it is a noteworthy fact that in mammals satellite cells appear to be preferentially distributed near innervation sites\(^{60}\). This
strategic position suggests that neurotrophins and other soluble nerve-derived trophic factors are likely to affect satellite cell behavior particularly in mammals. In line with this, long-term denervation causes a dramatic decline in satellite cell number, due to reduced mitotic capability and to increased apoptosis. It has also been found that satellite cells control motor neuron growth-cone and guide axon terminals to contact specialized sites on the post-synaptic cell. The importance of this close relationship is even more evident during the denervation process. After acute denervation, satellite cell number increases, similarly to what happens during muscle injury.

4.2.3. Vascular cells

Blood vessels (Fig. 6) establish a close association with the skeletal muscle tissue, and it has been observed that satellite cells are often localized in the proximity of capillaries in adult skeletal muscle. The vascular cells represent an important source of growth factors that interact with satellite cells, stimulating their activation, proliferation and self renewal. This communication is not only meant to favor skeletal muscle regeneration, but also to accomplish the angiogenesis process, which goes in parallel with tissue reconstruction. Among these soluble factors, some of them have been already described as components of the immediate niche as well (IGF-1, HGF, FGF). Besides these, the vascular endothelial growth factor (VEGF) is another molecule released by endothelial cells, promoting myoblast proliferation, differentiation and preventing apoptosis.

4.3. The “systemic milieu”

Unlike the immediate and local milieu, components of the systemic milieu, such as the circulating hormones or immune cells, are located away from the skeletal muscle. Hence, the systemic milieu triggers a quite delayed satellite cell response.
4.3.1. **Immune cells**

In resting conditions, only a small percentage of immune cells resides in the local milieu of adult skeletal muscle, including mast cells and macrophages. Upon muscle injury, these cells act as stress-sensors and secrete chemoattractive molecules, allowing the infiltration of other immune cells from the systemic environment. This results in a temporary migration of systemic immune cells to the local milieu of the satellite cell niche. Although the mechanisms through which immune cells establish an interplay with muscle cells is not completely clear, *in vitro* experiments demonstrated that macrophages potentiate myoblast activation, proliferation, migration and that myoblasts in turn secrete cytokines and growth factors to potentiate immune cell infiltration\textsuperscript{64,65}.

5. **The skeletal muscle regeneration during ageing**

At the skeletal muscle level, aging is associated with decrease in muscle mass, in strength and endurance, with a consequent loss of functional mobility. The term “sarcopenia” (from Greek, “poverty of flesh”) is generally used to describe all the abovementioned age-related changes of the skeletal muscle. It was estimated that by 60-70 years of age, muscle mass decreases up to 25-30% in humans\textsuperscript{66}. Aged muscles display a smaller myofiber cross-sectional area (CSA), an increased fibrosis and a longer time to recover after injury. In addition, the gradual age-associated denervation leads to a remodeling of motor units, with changes in fiber-type composition\textsuperscript{67,68}. The pathophysiological implications of aging are worsened by low physical activity and training of old people\textsuperscript{68}. Immobilized aged muscle biopsies revealed a status of myofiber degeneration comparable to severe pathological conditions, together with a chronic status of inflammation.

The decline in neuromuscular function and performance is partially due to an impaired ability of myogenic precursors to contribute to tissue turnover and repair. The wide range of factors determining the less successful tissue regeneration in aged muscles includes intrinsic and extrinsic changes. Intrinsic mechanisms consist in reduction and alteration of
satellite cell proliferative potential, while extrinsic factors comprise the altered expression and release of local/systemic growth factors and cytokines.

5.1. **Intrinsic factors**

Satellite cells have a limited proliferative capacity, mainly due to telomere shortening. This is particularly evident in human cells that, unlike the murine ones, do not express telomerase\(^69\). In fact, cultured primary human cells display an initial active proliferation, rapidly declining after consecutive *in vitro* passages, till the proliferative senescence\(^70\). In addition to the reduced ability to duplicate, the senescence phase is also characterized by myoblast reduced mobility, capacity to fuse and to properly differentiate\(^70,71\).

In humans, a reduction in satellite cell proliferative ability is observed during childhood. Neonatal and infant satellite cells are capable of more than 45 divisions, while a dramatic decline in the number of cycling rounds occurs after 9-15 years of age\(^70\)(Fig. 7). The direct consequence is the reduction of available precursors and of their capacity to regenerate the myofiber population.

However, contrasting estimations of aged satellite cell number are reported in literature. Moreover, some Authors provided evidence that the intrinsic proliferative potential, even if reduced in aged satellite cells, should be high enough to allow an efficient regeneration of skeletal muscle tissue throughout the entire adulthood\(^71,72\). Thus, the role of intrinsic factors in determining the impaired regenerative potential of satellite cells remains still matter of debate.

5.2. **Extrinsic factors**

Aging compromises the physiological functions of all organs in the body, and therefore leads to changes in the release of vascular, neural, interstitial and systemic factors. Accumulating evidence points out that age-specific changes depend mostly on the “age” of the extracellular milieu, rather than on the satellite cell per se\(^73\). Transplanting experiments of old muscles into young animals, during which satellite cell regeneration was successfully restored represent the strongest evidence for this theory\(^74\). Nowadays, the emerging theory is that the diminished regenerative potential of aged muscle is mostly due to epigenetic modifications. These changes would reflect alterations of the
aged environment, making the niche less permissive and sub-optimal for satellite cell proliferation and differentiation\textsuperscript{75,76}. Even the efficiency of the inflammatory response is compromised by aging and immune cells with their relative secreted factors are significantly reduced in the satellite cell local milieu\textsuperscript{77}. Other reasons that could drive to the generation of this sub-optimal environment are the thickening of the basal lamina, the increased fibrosis and the reduced capillary density.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{satellite_cell_figure.png}
\caption{Graph and table describe satellite cell life-span and cumulative number of divisions in subjects of different ages. In newborns (up to 5 months), satellite cells display a high cycling activity and the total number of divisions ranges from 46 to 65. After childhood (9-15 years), satellite cells show a decreased proliferative capacity and experience an earlier cellular senescence (from \textsuperscript{70}).}
\end{figure}
AIM OF THE THESIS
As reported in the section “Introduction”, satellite cells are skeletal muscle myogenic precursors that sustain regeneration and repair of the tissue during post-natal life. Their behavior is significantly affected by physical, mechanical and chemical cues related to their surrounding environment.

The main goal of this thesis was to investigate the effects of some of the trophic factors, normally released in vivo in the immediate niche and/or in the local milieu, to further characterize their role in the modulation of satellite cell functions and homeostasis. Experiments have been performed on satellite cells isolated either from mice and/or from humans, taking into account possible species-specific effects of the chemical signals of interest.

In each of the following sections, before going into details to different research works and results, it will be provided a general overview of the main functional aspects of the trophic factors analyzed.
6. The role of ATP and adenosine in the regulation of myoblast proliferation and myotube differentiation

ATP is an important signaling molecule released extracellularly by many cell types under resting conditions, or after pathological events. Through its autocrine/paracrine signaling, ATP modulates many biological functions like neurotransmission in the central and peripheral nervous systems, smooth muscle contraction, immune response, platelets aggregation, bone remodeling and more in general growth, proliferation and apoptosis\(^7\). At the skeletal muscle level, ATP is released by myofibers in response to the contractile activity\(^7\) and by the motor nerve endings during the cholinergic transmission\(^8\).

ATP effects are mediated by purinergic receptors, that are divided into two classes, ionotropic (P2X)\(^8\) and metabotropic (P2Y)\(^8\). Evidences show that purinergic receptors are expressed by activated satellite cells and this suggests the possible role of extracellular ATP as a trophic factor involved in the regulation of tissue regeneration\(^8\).

During the myogenic program, different P2X and P2Y isoforms are expressed in the proliferation and differentiation phases. However, in myoblasts only few receptor subtypes are present, while most isoforms are upregulated in differentiating myotubes\(^8\). This fact suggests that ATP may display a different role according to the stage of the myogenic program. Martinello and colleagues have proposed ATP as one of the controllers of myoblast proliferation\(^8\).

Interestingly, extracellular ATP stimulation of purinergic receptors has been suggested to promote the production of reactive oxygen species (ROS) in the skeletal muscle\(^8\). Although ROS are associated with cell damage, cell death and with the pathogenesis of many diseases\(^9\), increasing data indicate that at low concentrations ROS, and particularly H\(_2\)O\(_2\), play an important role as second messengers, positively regulating several cell functions. Regardless to the skeletal muscle, ROS are considered key modulators of muscle physiological functions, such as contraction, development, metabolism and blood flow\(^10\).

In this scenario, one of the main questions addressed in this thesis was to investigate if ATP might act as an extracellular trophic factor controlling ROS production in proliferating myoblasts. The results obtained confirmed our working hypothesis (6.1. Appendix 1 – Published results).
It is also important to consider that extracellular ATP has generally a short half-life because of its fast breakdown by multiple families of ecto-nucleotidases, located at the cell surface or in the extracellular matrix. Due to a different expression of ecto-enzymes during skeletal muscle regeneration, resting levels of extracellular ATP vary from myoblasts to myotubes: in the former the nucleotide's concentrations are stably high, whereas in the latter it is quickly hydrolyzed because of an increased ecto-ATPase expression with differentiation\(^8\). The result is the prevalence of signaling molecules such as AMP and adenosine in the microenvironment of differentiating skeletal muscle cells in the later phases of myogenesis.

In the skeletal muscle, among the purine nucleosides deriving from ATP, adenosine is well-known to be an important modulator of blood flow\(^8\) and of insulin-mediated glucose uptake\(^9\) in the skeletal muscle tissue. Moreover, adenosine derived from the hydrolysis of ATP released during the cholinergic signaling, mediates a depressant action in the neuromuscular transmission, by inhibiting ACh release from the presynaptic terminal\(^9\)\(^0\)\(^1\).

The expression of adenosine receptors in activated myogenic precursors suggests that extracellular adenosine, acting in autocrine or paracrine fashion, could have an impact on satellite cell properties\(^9\)\(^2\). Therefore, it is conceivable that, besides the well known physiological roles of the nucleoside in the cell physiology of the skeletal muscle fibers, the range of functions might be extended also to the regenerative process and to the control of the properties of myogenic cells.

Considering the highest level of adenosine in the microenvironment after satellite cell activation, it is reasonable that extracellular adenosine might play a prevalent role in myotube formation and/or maturation. In this context, we have also investigated the potential role of adenosine during skeletal muscle regeneration. Preliminary results suggest that adenosine modulates the nAChR-driven intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) spikes in differentiated myotubes, indicating a novel role for adenosine in the differentiation of skeletal muscle cells and regeneration of the skeletal muscle tissue (6.2. Appendix 2 – Preliminary results).
APPENDIX 1 – PUBLISHED RESULTS
Original Contribution

Reactive oxygen species contribute to the promotion of the ATP-mediated proliferation of mouse skeletal myoblasts

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A B S T R A C T

Reactive oxygen species (ROS) and extracellular adenosine 5′-triphosphate (ATP) participate in autocrine and paracrine regulation in skeletal muscle. However, the link between these two signaling systems is not well established. Here, we studied cell proliferation as a possible consequence of the trophic effect of ATP in cultured skeletal muscle myoblasts and we tested the possibility that low concentrations of ROS represent the intermediate signaling molecule mediating this effect. Exposure to 10 μM ATP increased proliferation of mouse myoblasts by ~20%. ATP also induced intracellular Ca2+ oscillations, which were independent of extracellular Ca2+. Both effects of ATP were prevented by suramin, a broad-spectrum purinergic P2 receptor antagonist. In contrast, the adenosine receptor blocker CGS-15943 did not modify the ATP-mediated effects. Consistent with this, adenosine per se did not change myoblast growth, indicating the direct action of ATP via P2 receptor activation. The proliferative effect of ATP was prevented after depletion of hydrogen peroxide (H2O2) by the peroxidase enzyme catalase. Low-micromolar concentrations of exogenous H2O2 mimicked the stimulatory effect of ATP on myoblast growth. DCF imaging revealed ATP-induced catalase and DPI-sensitive ROS production in myoblasts. In conclusion, our results indicate that extracellular ATP controls mouse myoblast proliferation via induction of ROS generation.

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Introduction

At the developing neuromuscular junction, pre- and postsynaptic effects of adenosine 5′-triphosphate (ATP) have been described owing to the activity of purinergic P2 receptors [1]. ATP is stored together with acetylcholine [2,3] and co-released by motoneurons. Moreover, ATP is also released by muscle fibers in the extracellular environment, after mechanical distortion or contraction [4], electrical activity [5], membrane depolarization, or hypoxia. It acts on muscle cells as an autocrine or paracrine signal, if coming from neighboring cells, and exerts a wide range of cellular effects acting on P2X and P2Y purinergic receptors [6]. Recent evidence reveals that low levels of extracellular ATP have a physiological effect also on myoblasts, the skeletal muscle precursor cells [7,8], as well as on muscle remodeling [9] in response to chemical and physical stimuli. Because of the low extracellular ATP-hydrolyzing activity of the muscle myoblast environment, ATP activity might be persistent and suggested not to decay easily [8] and thus is potentially able to act as a trophic factor. In line with this, purinergic receptors are found in skeletal myoblasts [8,10] and their expression is strictly regulated during development and after injury [11]. In this scenario, ATP has been suggested as a potential modulator of the regenerative capacity of satellite cells, the resident myogenic cells responsible for muscle adaptation in response to various stimuli, such as physical exercise, and for tissue regeneration after injury [12,13].

At the neuromuscular junction, reactive oxygen species (ROS) contribute to the presynaptic action of ATP [14] and, in preparations different from muscle, extracellular ATP has been hypothesized to act via redox mechanisms. In astrocytes [15] and glioma cells [16], ATP-mediated purinergic receptor activation is sufficient to induce ROS production. In central nervous system synapses, in immature hippocampus, ATP-mediated potentiation of GABAergic synaptic transmission is mediated by ROS [17]. However, there is no evidence available on ROS-mediated ATP action in myoblasts.

The goal of our research was therefore to investigate if ROS are part of the signaling pathway used by low physiological levels of ATP, working at purinergic receptors on the membrane of muscle cells. Our results demonstrate a novel trophic effect of ATP on the
growth of mouse myoblasts and suggest the redox agent hydrogen peroxide (H₂O₂) as an important intermediate downstream of ATP receptor pathways leading to enhanced cell proliferation of muscle precursors. New findings related to the physiological effects of ROS open new perspectives for the comprehension of skeletal muscle excitability, plasticity, and regeneration.

Materials and methods

Cell culture

Primary myoblast cell cultures were established from mouse satellite cells, kindly provided by Professor A. Wernig (University of Bonn; for details see [18]). Briefly, cells were isolated from the hindleg of 7-day-old male BALB/c mice killed by cervical dislocation as approved by the local Animal Care Committee and in agreement with European legislation. Muscle tissue was minced and then enzymatically dissociated with collagenase and trypsin. Expansion and enrichment of myoblasts were achieved and experiments were performed on cell cultures showing a myogenic purity higher than 90%, tested by desmin immunofluorescence staining, as previously described [19]. Myoblasts were maintained as exponentially undifferentiated cells [20] in growth medium (GM) consisting of Ham’s F-10 supplemented with 20% fetal calf serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and subcultured by standard trypsinization every 3 days. Cultures were grown at 37 °C and 5% CO₂.

Cell proliferation and fusion

To evaluate cell proliferation, subcultured undifferentiated myoblasts at a density of 3 × 10^5 cells in 35-mm-diameter petri dishes were exposed to various stimuli 24 h after seeding in GM. Proliferation was measured 3 days after stimulation. The proliferative capacity was expressed as mean percentage increase in cell number with respect to controls (unstimulated cells), from at least three independent experiments performed in triplicate.

To induce cell differentiation and fusion, mononucleated myoblasts were plated at a density of 70 × 10^3 cells on 24-mm coverslips coated with Matrigel. Twenty-four hours after plating, the GM was replaced with differentiation medium (DM) consisting of Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum and L-glutamine, penicillin, and streptomycin as for GM. DM was renewed every 2 days. The myoblast differentiation efficiency was quantified after 6 days in DM by evaluating the fusion index, defined as the number of myotube nuclei vs the number of total nuclei. Myogenic cells were labeled with desmin immunofluorescence staining, marking the nuclei with bisbenzimide H-33258 [19]. Quantification of nuclei was performed from at least 10 randomly chosen optical fields per coverslip for four independent experiments.

Calcium imaging

Intracellular calcium concentration ([Ca²⁺]₀) was monitored using the fluorescent Ca²⁺ indicator fluo-2-pentaetoxymethyl ester (Fura-2 AM). Myoblasts were plated on Matrigel-coated coverslips and loaded with the Ca²⁺ indicator Fura-2 AM (5 μM, 30 min in the dark) at room temperature, in a normal external solution (NES; see below) supplemented with 1 mg/ml bovine serum albumin. After washout, the cells were maintained in NES for 15 min at room temperature to allow deesterification of the dye. A temperature-controlled microincubator chamber (Medical Systems Corp.; Davie, FL, USA) kept the temperature at 37 °C during the experiments. Cells were excited alternately at 340 and 380 nm, selected by a monochromator (Polychrome II Till Photonics GmbH, Martinsried, Germany) and fluorescence signals were collected by a CCD camera (SensiCam; PCO Computer Optics, Kelheim, Germany) at an image acquisition rate of 1 ratio/s. The monochromator and CCD camera were controlled by Till Vision software (Till Photonics), also used for image processing. The ratio of fluorescence images (340/380) and the corresponding temporal plots (i.e., variations in the mean value of the fluorescence intensity obtained from regions of interest) were calculated offline. In the temporal plots, the fluorescence ratio at rest was assumed to be 1. Variation in the [Ca²⁺]₀ was expressed in the plots as increase in fluorescent signal relative to the fluorescence at rest. Each set of experiments was carried out in at least three different cell preparations. The number of analyzed cells for each experimental point exceeded 40.

ROS measurement

Myoblasts were plated into black optical-bottom 96 wells, at a density of 12.5 × 10⁴ cells/well. ROS measurements were carried out 24 h after plating as previously described [21]. Briefly, cells were loaded with 2 μM 5-(and-6)-carboxy-2’,7’-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA; Invitrogen, Carlsbad, CA, USA) for 30 min at 37 °C in Hepes-buffered salt solution. After washout, the cells were acutely stimulated with 10 μM ATP and immediately analyzed online for ROS generation. In some experiments myoblasts were preincubated with diphenyleneiodonium chloride (DPI; 25 μM, 60 min) or catalase (1200 U/ml, 15 min). H₂O₂ (3 μM) was used as reference control.DCF in vivo fluorescence imaging was assessed using a fluorimeter (PerkinElmer, Waltham, MA, USA) every 5 min up to 30 min at 37 °C. Fluorescence was expressed as F/F₀, where F was the fluorescence signal after 15 min and F₀ the baseline fluorescence value of the same wells before stimulation. Mean changes in fluorescence were expressed as percentage of control. For each stimulus, ROS measurement was performed in triplicate, and the data represent the means of three independent experiments.

Cell viability

Cell viability was assessed by 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyltetrazolium bromide (MTT [22]) and sulforhodamine B (SRB [23]) assays. Cells were seeded into 96-well plates at 12.5 × 10⁴ cells/well in GM. After 24 h, the cells were exposed to 3 or 100 μM H₂O₂ for 30 min, then the H₂O₂ was washed out and GM was renewed. MTT and SRB assays were performed 24 h after stimulation.

For MTT assay, cells were incubated for 5 h in 20 μl/well of a 5 mg/ml MTT solution. The medium was then removed by vacuum aspiration, and cellular formazan crystals were dissolved with dimethyl sulfoxide (200 μl/well). Absorbance was measured on an Automated Microplate Reader EL 311s (Bio-Tek Instruments, Winooski, VT, USA) with a reference wavelength of 630 nm and a test wavelength of 540 nm.

For SRB assay, cells were washed twice with phosphate-buffered saline (PBS; 100 μl/well) at room temperature and then fixed with a 50% trichloroacetic acid solution (50 μl/well) for 60 min at 4 °C. After 1 h, the wells were rinsed twice with bidistilled water (100 μl/well) and incubated with SRB (0.4% in 1% acetic acid, 100 μl/well) for 30 min at room temperature. After three washouts with 1% acetic acid (100 μl/well) to eliminate colorant excess, the cells were solubilized with tris[hydroxy-methyl]aminomethane (10 mM, 200 μl/well). Sample absorbance was read on the Automated Microplate Reader EL 311s with a single wavelength of 570 nm. MTT and SRB assays were performed at least three times in 15 replicate wells for each H₂O₂ concentration.
Solutions and chemicals

Freshly prepared hydrogen peroxide was diluted in GM from a 40% (w/v) stock solution. The physiological solution (NES), PBS, and Hepes-buffered salt solution (HBSS) contained (in mM): NES, NaCl 140, KCl 2.8, CaCl₂ 2, MgCl₂ 2, glucose 10, Hepes 10 (pH adjusted to 7.35 with NaOH); PBS, Na₂HPO₄ 10; NaH₂PO₄ 1.8, NaCl 145; and HBSS, NaCl 120, KCl 5.4, CaCl₂ 1.8, glucose 10, NaHCO₃ 25, Hepes 25. Media, horse serum, antibiotics, and ♂-glutamine were purchased from Sigma (St. Louis, MO, USA). Fetal calf serum was from Mscia Brunelli (Milan, Italy), Matrigel from Becton–Dickinson (Rutherford, NJ, USA), and CGS-15943 from Tocris Bioscience (Bristol, UK). All the other chemicals, unless otherwise stated, were from Sigma.

Data acquisition and statistical analysis

All data are presented as the means ± SE with n being the number of petri dishes, unless otherwise stated. GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA) software was routinely used for statistical analysis and graph plotting. Differences between data were evaluated by one-way ANOVA, followed by Bonferroni’s post hoc test.

Results

Extracellular ATP affects myoblast proliferation

To test the possible role of extracellular ATP on muscle satellite cell proliferation, primary cultures of mouse myoblasts maintained in GM as undifferentiated exponentially growing cells were exposed to various doses of the nucleotide. Fig. 1 shows that the growth rate of mouse myoblasts was significantly increased only by chronic treatment with 1 or 10 µM ATP (respectively 26.37 ± 5.77%, n = 10, and 22.60 ± 2.29%, n = 68, increase with respect to the controls; p < 0.05) at day 4. Interestingly, even after a single and short exposure (30 min, data not shown) to 10 µM ATP we still observed the stimulatory effect of the nucleotide on myoblast proliferation (29.10 ± 3.66%, n = 27, p < 0.05, data not shown). In myoblasts treated chronically with very low (0.1 µM) or relatively high (100 µM) concentration of ATP, the cell number at day 4 did not change significantly compared with controls (−0.63 ± 5.41%, n = 5; 4.70 ± 2.91%, n = 8, respectively). Interestingly, very high concentrations of ATP (1 and 3 mM) significantly reduced the proliferative capacity of myoblasts in a dose-dependent manner (−45.6 ± 2.33%, n = 5; −95.7 ± 1.06%, n = 6; of control, respectively, p < 0.05).

The ATP-induced myoblast proliferation is mediated by P2 receptor activation

Preincubation (15 min) with 100 µM suramin prevented the effect of 10 µM ATP on cell proliferation. The number of myoblasts was −8.46 ± 3.59% of controls (n = 10), significantly different from that observed in cells treated with ATP alone (19.61 ± 3.19% of controls, n = 15; p < 0.01). Treatment with 100 µM suramin alone, however, did not significantly change myoblast proliferation (−8.66 ± 4.97%, n = 14; Fig. 2A). To exclude the possibility that the mitogenic effect of 10 µM ATP was mediated by adenosine, we also explored the direct effect of this ATP derivative on myoblast proliferation. However, the equimolar (10 µM) concentrations of adenosine did not affect cell proliferation (−0.93 ± 1.85% of controls, n = 9; p > 0.05). In addition,
the broad-spectrum adenosine receptor antagonist CGS-15943 (100 nM) did not inhibit the effect of 10 μM ATP on cell proliferation (18.03 ± 2.39%, n = 10 vs 21.80 ± 3.13% in ATP alone, n = 15; p > 0.05; Fig. 2B). These results suggested that the trophic effect of ATP on myoblast proliferation was triggered by activation of P2 receptors.

To investigate the extracellular ATP signaling pathway, Ca²⁺ imaging experiments were performed. Superfusion of 10 μM ATP induced spontaneous [Ca²⁺], oscillations in the majority of cultured mouse myoblasts tested (85.65 ± 6.04%, n = 5). Fig. 3A illustrates typical ATP-induced Ca²⁺ oscillatory patterns. In the presence of ATP alone, [Ca²⁺], responses progressively decreased in amplitude. A complete recovery of basal [Ca²⁺], was thus observed within 5 min of the onset of the oscillatory activity (mean 299.42 ± 12.59 s, n = 51 cells). The addition of the Ca²⁺ chelator EGTA (5 mM) to the bathing medium did not prevent ATP-induced Ca²⁺ oscillations. The number of ATP-responsive cells was not affected (80.25 ± 8.24%, n = 5, Fig. 3B), indicating that the ATP-induced Ca²⁺ response did not depend on Ca²⁺ influx.

Preincubating the cells with the broad purinergic P2 receptor antagonist suramin (100 μM), however, drastically reduced the number of cells responsive to ATP (1.00 ± 0.29%, n = 3), confirming the direct action of ATP via P2 receptors. All these data suggest a prevalence of purinergic P2Y receptors in proliferating myoblasts.

ATP induces ROS generation

Because the action of ATP in adult muscle is mediated by the induction of ROS [17], we next tested if the action of ATP was ROS-dependent also in proliferating myoblasts. To directly investigate whether ATP triggered ROS generation, we utilized the fluorescence probe carboxy-H₂DCFDA, commonly used to detect ROS formation in living cells [24]. Myoblasts exposed to 10 μM ATP exhibited a significant and fast change (33.68 ± 7.68%, p < 0.01, 15 min after stimulation) in the fluorescent signal, indicating a fast increase in endogenous ROS. Interestingly, a similar (34.77 ± 15.53%) increase in fluorescence was observed after adding 3 μM H₂O₂ as a positive control (Fig. 4A, p < 0.05). Furthermore, cell preincubation with the peroxide scavenger catalase (1200 U/ml, 15 min) prevented the ATP-induced ROS production (p < 0.05).

The role of NADPH oxidase as a possible source of ROS elicited by ATP was also investigated. To inhibit the endogenous NADPH oxidase, myoblasts were preincubated with DPI (25 μM) for 60 min. Pretreatment with DPI blocked the ATP-elicted increase in intracellular fluorescence (Fig. 4A, p < 0.01). These results indicate that in cultured myoblasts the NADPH oxidase complex was involved in the generation of ROS as an intermediate of ATP-mediated signaling.

Role of ROS in the trophic effect induced by ATP

A set of experiments was planned to investigate if ROS promoted the ATP-induced myoblast proliferation. Preincubation (15 min) with the H₂O₂ scavenger enzyme catalase (1200 U/ml) prevented the ATP-mediated cell proliferation (−5.94 ± 3.29% vs 22.28 ± 7.04% in ATP, n = 13; p < 0.001; Fig. 4B) without any effect on cell number per se (−2.86 ± 4.28% of control, n = 15; p > 0.05). In parallel, myoblasts were exposed to various concentrations of H₂O₂ (0.1, 3, 30, and 100 μM) and a bell-shaped concentration-dependent effect of H₂O₂ on myoblast proliferation was observed. The lowest efficacious concentration

Fig. 3. [Ca²⁺], responses induced by ATP. (A) Representative spontaneous [Ca²⁺], oscillatory patterns observed under three different experimental conditions: in the presence of 10 μM ATP alone, with 5 mM EGTA, and with 100 μM suramin. Ordinates represent the fluorescence increase relative to the value at rest. (B) Percentage of responsive cells under the three different experimental conditions shown in (A). ATP induced [Ca²⁺], oscillations in similar numbers of cells both in the presence and in the absence (5 mM EGTA) of external Ca²⁺, whereas suramin prevented the ATP-induced [Ca²⁺], response. *p < 0.05, significantly different from the ATP-alone-treated cells.
detected protein content that was significantly \( p < 0.05 \) higher than in controls, whereas no significant effect was observed in cell cultures treated with 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) \( \text{(p} > 0.05; \text{data not shown).} \) The MTT assay excluded any toxic effect on cell viability at both \( \text{H}_2\text{O}_2 \) concentrations \( \text{(data not shown).} \) These data confirmed the enhancing effect of 3 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) on myoblast proliferation and excluded any toxic effect of \( \text{H}_2\text{O}_2 \) in the concentration range used for our experiments.

Nevertheless, under the same conditions \( \text{H}_2\text{O}_2 \) did not affect myoblast differentiation as demonstrated by unaltered differentiation efficiency, evaluated by calculating the fusion index at 6 days in culture, in controls and in cultures treated for 30 min with 3 and 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \). The fusion index values revealed that neither \( \text{H}_2\text{O}_2 \) concentration affected the ability of myoblasts to form multinucleated myotubes \( \text{(controls} 20.88 \pm 1.84\% ; 3 \mu \text{M} \text{H}_2\text{O}_2 \text{18.21} \pm 2.46\%; 100 \mu \text{M} \text{H}_2\text{O}_2 \text{24.50} \pm 2.22\%; \text{data not shown).} \) The unaltered fusion index seemed to exclude a side effect of low concentrations of \( \text{H}_2\text{O}_2 \) on differentiation capability of myoblasts and confirmed that \( \text{H}_2\text{O}_2 \) was not toxic in the concentration range used.

**Discussion**

Despite its well-known role as an intracellular energy source, ATP released by skeletal muscle during electrical activity [5] could induce short-term or long-term changes in the muscle by acting on membrane purinergic receptors. ATP might therefore work as an intercellular signaling molecule, favoring muscle regeneration via the proliferation and differentiation of satellite cells [25].

This study reports for the first time a ROS-mediated stimulatory effect of extracellular ATP on the proliferation rate of mouse skeletal myoblasts derived from satellite cells.

**ATP promotes proliferation of skeletal myoblasts**

We observed that physiological levels of extracellular ATP (\( \sim 1–10 \mu \text{M} \)) [9,26,27] induce a more than 20\% increase in proliferation of mouse myoblasts. A lower concentration (0.1 \( \mu \text{M} \)) was not enough to be effective, whereas higher doses (1 and 3 \( \mu \text{M} \)) reduced cell growth. We can argue that at high concentration, ATP can operate via different purinergic receptors, triggering different functional effects. In the concentration range promoting cell proliferation, our results indicate that ATP acts via suramin-sensitive P2 receptors. The expression of P2 receptors in skeletal myoblasts was previously reported by several groups [8,28,29].

In proliferating myoblasts, under our experimental conditions, application of ATP elicited [\( \text{Ca}^{2+} \)] oscillations, which were independent of external \( \text{Ca}^{2+} \), indicating a cell signaling based mainly on \( \text{Ca}^{2+} \) release from internal stores. This effect is consistent with the involvement of metabotropic P2Y receptors, which are normally coupled with inositol trisphosphate (IP3)-dependent \( \text{Ca}^{2+} \) release from intracellular reservoirs.

Effects of ATP on cell proliferation have been previously reported, even though conflicting results were obtained. Recently, the proliferative action of ATP via P2X and P2Y receptors was found in C2C12 skeletal myocytes maintained in differentiation medium [8]. However, in primary cultures and in C2C12 muscle cells, extracellular ATP at relatively high 100 \( \mu \text{M} \) concentration inhibited proliferation, favoring myoblast differentiation into myotubes [10,30]. In addition, in primary cultures, 10 \( \mu \text{M} \) ATP did not inhibit cell proliferation in a serum-enriched medium, in which immunoreactivity for P2X was not detected [10]. Moreover, the same authors reported that in general, P2Y receptor agonists did not inhibit myoblast proliferation. In particular, and in accord with our suggestions on P2Y-promoted proliferation,
UTP, a specific agonist of P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub> receptors, increased the skeletal myocyte myocyte number [8,10]. The apparent discrepancy in the action of extracellular ATP on myoblast proliferation could therefore be explained by the fact that various purinergic receptor subtypes could be expressed in different muscle cell lines as well as under different serum conditions.

The possibility that the ATP derivatives ADP and AMP could be contributing to the observed positive ATP-related changes in myoblast proliferation was also considered; however, it is known that cultured myoblasts have low ATP-hydrolyzing activity by ecto-ATPases, which is suggested to preserve cell viability because AMP and ADP were found to show cytotoxic effects in C2C12 cells [8]. We also demonstrated that the ATP-mediated proliferation was not due to the conversion of ATP into its principal end metabolite adenosine. In neither case did the adenosine receptor antagonist CGS-15943 affect the ATP-mediated myoblast proliferation, and adenosine per se did not alter the growth rate of proliferating myoblasts, as already observed by other groups [10].

**ROS-dependent action of purines in skeletal muscle**

ROS are also produced by skeletal muscle during exercise [31]. Here, we tested whether ATP signaling leading to enhanced myoblast proliferation is mediated by a ROS-dependent mechanism. Three lines of evidence are consistent with such a mechanism. First, ROS imaging with the H<sub>2</sub>O<sub>2</sub>-sensitive dye DCF revealed that exogenous ATP induced ROS generation. The similar intracellular fluorescence increase induced by 3 μM H<sub>2</sub>O<sub>2</sub> is consistent with the fact that in mouse myoblasts purinergic receptor activation allows the production of peroxides at comparable low-micromolar concentrations. Second, the proliferative effect of ATP was prevented after breaking down of extracellular H<sub>2</sub>O<sub>2</sub> by the membrane-impermeable catalase. Our results are consistent with previous experiments suggesting that the enzyme, even if added extracellularly, controls the intracellular redox status, because H<sub>2</sub>O<sub>2</sub> can freely diffuse across cell membranes [32]. Third, low-micromolar concentrations of exogenous H<sub>2</sub>O<sub>2</sub> mimicked the stimulatory effect of ATP on myoblast growth. A similar stimulatory effect was also observed in fibroblasts, bacteria [33], yeast cells [34], and pulmonary artery endothelial cells [35]. Thus, H<sub>2</sub>O<sub>2</sub> represents a key signaling molecule during myogenesis [36], able to promote muscle cell regeneration.

The main sources of ROS in mammalian cells are mitochondria and oxidases, including NADPH oxidase. Recent studies show that H<sub>2</sub>O<sub>2</sub> generated after activation of the endogenous NADPH oxidase, favors the proliferation of myoblasts [37]. Furthermore, ROS produced by NADPH oxidase mediated mitogenic factor signaling pathways in leiomyoma cells [38].

In line with these results, our data indicate that the intracellular mechanisms coupling activation of purinergic receptors and H<sub>2</sub>O<sub>2</sub> generation in mouse skeletal myoblasts involve NADPH oxidase activation. The specific target for H<sub>2</sub>O<sub>2</sub>-mediated mitogenesis in skeletal myoblasts has not yet been found; plasma membrane receptor tyrosine kinases [39] as well as ion channel conductances [40] can be potentially involved.

**Conclusions**

In line with previous studies, such as the regulation of synaptic transmission at peripheral and central synapses [14,17], the promotion of tumor growth [16], and the modulation of macrophage immune responses [41], our results show a ROS-mediated action of extracellular ATP.

Here, a link between the activation of purinergic receptors by physiological levels of extracellular ATP, generation of NADPH oxidase-derived ROS, and potentiation of regenerative capability of skeletal muscle cells is revealed. Our data therefore support the intriguing hypothesis that ROS, well known to induce tissue damage under oxidative stress conditions (massive exercise, aging, and muscle disease), might promote muscle plasticity and regeneration if generated at low concentrations.

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Adenosine receptors affect the nAChR-driven \([\text{Ca}^{2+}]_i\) spikes in differentiating myotubes

Introduction

During myogenesis, differentiating rodent skeletal muscle cells display a contractile activity in the absence of innervation. It was already demonstrated that these twitches are sustained by \([\text{Ca}^{2+}]_i\) spikes that mainly depend on the upstream autocrine activity of the nAChRs. In fact, differentiating skeletal muscle cells synthesize and release ACh-like compounds. It has also been demonstrated that such nAChR-driven \(([\text{Ca}^{2+}]_i)\) spikes represent a crucial mechanism through which the differentiating muscle cell maintains its own trophism before innervations.

Adenosine signaling occurs through the activation of purinergic P1 receptors, named adenosine receptors (ARs). They represent a family of four G-protein coupled receptors, A1, A2A, A2B, A3, displaying different expression patterns and affinity for adenosine. Among the activities mediated by the ARs, there are those involving the modulation of the metabotropic and ionotropic neuroreceptors at the central and peripheral synapses. At the skeletal muscle level, very little is known about the expression and action of ARs, both during development and adult age. Interestingly, in rat myotubes, ARs were proposed to modulate nAChR channel activity. However, the role of adenosine in controlling the nAChR-driven \(([\text{Ca}^{2+}]_i)\) spikes has never been studied.

Taking into account all these observations, we have performed an analysis of the AR-mediated effects on the nAChRs-driven spontaneous \([\text{Ca}^{2+}]_i\) spikes and on the autocrine nAChR channel opening activity in in vitro differentiated mouse myotubes.
Methods

Cell culture

The experiments were performed on myotubes derived from the in vitro fusion and differentiation of expanded primary mouse myoblasts (called i28), kindly supplied to us by Dr A. Wernig, Department of Physiology and Medical Policlinic, University of Bonn, Germany. Briefly, these myogenic cells were plated at a density of 70000 cells either in 24 mm Matrigel-coated coverslips, or in 35 mm Matrigel-coated Petri dishes. Cells were maintained in proliferative conditions in the presence of HAM'S F-10 growth medium, plus 20% fetal bovine serum (FBS), l-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml). To induce cell differentiation and fusion into myotubes, 1 day after plating the growth medium was replaced with a differentiation medium, consisting of DMEM supplemented with 2% horse serum and l-glutamine, penicillin and streptomycin. The differentiation medium was renewed every 3 days to avoid loss of nutrients and growth factors. Cultures were maintained at 37 °C in a humid air atmosphere containing 5% CO₂.

Videoimaging

Videoimaging experiments were carried out as described in detail in Appendix I. During [Ca²⁺]ᵢ measurements, drugs were gently applied to the bathing solution by loading appropriate volumes of concentrated solution into a 2-ml syringe connected to the micro-incubator chamber via a small tube.

Electrophysiological recordings

Single channel recordings in cell-attached configuration were performed, at room temperature, in 6- to 8 day differentiated myotubes, when cells were able to generate spontaneous mechanical contractions, as previously reported by our lab. Cells were bathed in a normal external solution (NES) containing (mM): NaCl (140), KCl (2,8), CaCl₂ (2), MgCl₂ (2), glucose (10), Heps (10), pH 7.4. The patch pipettes were made up of borosilicate glass and fire-polished to a final tip resistance of 5-8 MΩ when filled with the
same NES. Single channel currents were recorded using an Axopatch 200 amplifier. Signals were filtered at 1 kHz with a lowpass Bessel filter and analyzed post-acquisition by using the pCLAMP 8.0 software package (Axon Instruments).

Control and experimental data were collected immediately after achieving the gigaseal, when the single channel activity was stable. To examine with accuracy the channel open time, only traces with single openings were considered for analysis. Channel conductance was estimated from the slope of the regression line obtained by plotting the current amplitude against the pipette potentials (Vp) at 40, 60, and 80 mV. All kinetic analysis were performed at 60 mV pipette potential. The resting membrane potential was calculated under cell-attached configuration, by recording the activity of nAChR channels at different pipette potentials, assuming AChR reversal potential at 0 mV. Data were analysed using SigmaPlot software (OriginLab Corporation, Northampton, MA, USA) and are given as means ± SEM; data sets are considered statistically different when \( p < 0.05 \) (ANOVA Bonferroni’s post hoc test).

**Chemicals**

Fetal calf serum was purchased from Life Technologies (Monza MB, Italy) and Matrigel from Becton–Dickinson (Rutherford, NJ, USA). Adenosine ligands were purchased by Tocris Bioscience (Bristol, United Kingdom) and ADA was from Roche S.p.A. (Milano, Italy). All the other chemicals, unless otherwise stated, were from Sigma (St. Louis, MO, USA).
Results

AR ligands modulate the nAChR-driven [Ca\(^{2+}\)]\(_i\) spikes

As previously reported, autocrine nAChR activity activates the excitation-contraction coupling mechanism with the generation of [Ca\(^{2+}\)]\(_i\) spikes and contractions\(^{95}\). To verify whether the AR-mediated activity could modulate the embryonic nAChR channel openings, videoimaging experiments were performed in presence of non-specific AR ligands, namely the agonist NECA (100 \(\mu\)M), the stable adenosine analogue, and the antagonist CGS 15943 (100 nM). Calcium imaging experiments revealed that NECA increased the [Ca\(^{2+}\)]\(_i\) spiking frequency in 65.00 ± 10.00\% of cells observed (\(n = 5\) optical fields, 19 cells), and CGS 15943 completely abolished the activity in all the spiking cells analysed (\(n = 3\) optical fields, 8 cells), indicating that the AR-activity affected the functional phenomena driven by the nAChR (Fig. 1).

Fig. 1. Effect of NECA and CGS in nAChR-driven [Ca\(^{2+}\)]\(_i\) spikes. The ARs agonist NECA 100 \(\mu\)M induced an increase in the [Ca\(^{2+}\)]\(_i\) spiking frequency, while the ARs antagonist CGS 15943 blocked [Ca\(^{2+}\)]\(_i\) transients.
Endogenous adenosine promotes $[\text{Ca}^{2+}]_i$ spiking activity

To test whether the endogenous content of adenosine, released by contracting myotubes, was in the concentration range efficacious to modulate nAChR-driven $[\text{Ca}^{2+}]_i$ spiking activity, a series of experiments was carried out in presence of adenosine deaminase (ADA), the enzyme that catalyzes the irreversible deamination of adenosine to inosine, a molecule unable to activate ARs. Pre-incubation with ADA (U/ml, 60 min at RT) caused a significant reduction in the spontaneous $[\text{Ca}^{2+}]_i$ spikes of twitching myotubes. Specifically, ADA blocked or significantly inhibited the frequency of $[\text{Ca}^{2+}]_i$ spiking activity in $87.50 \pm 5.99\%$ of the cells observed ($n = 6$ optical fields; 21 cells; Fig. 2).

![Figure 2](image)

**Fig. 2.** Effect of the degradation of extracellular adenosine. ADA induced either a block or an inhibition the frequency of nAChR-driven $[\text{Ca}^{2+}]_i$ spiking activity. ADA was added to spiking myotubes and pre-incubated for 60 min before $[\text{Ca}^{2+}]_i$ measurements.

Role of the different AR subtypes on nAChR channel activity

In order to assess the specific role of each AR subtype, videoimaging experiments were performed on spiking myotubes treated with selective AR antagonists: ANR94 (100 nM) for the $A_{2A}\text{R}$, MRS1764 (20 nM) for the $A_{2B}\text{R}$, and ANR235 (100 nM) for the $A_3\text{R}$. While cell superfusion with inhibitors for $A_1\text{R}$, $A_{2A}\text{R}$ and $A_3\text{R}$ did not affect $[\text{Ca}^{2+}]_i$ spiking, the $A_{2B}$
antagonist MRS 1754 blocked the phenomenon in 66.67 ± 23.57% of the cells (n = 4 optical fields; 10 cells; Fig. 3).

**Fig. 3.** Effect of specific A2B Rs antagonist. The selective inhibition of the A2B R by 20 nM MRS 1754 induced a decrease in [Ca\(^{2+}\)]\(_i\) spiking activity of twitching myotubes.

**AR aspecific ligands modulate the nAChR-channel openings**

In order to explore AR modulation of the biophysical properties of the embryonic nAChR channel, another set of experiments aimed at recording single channel openings in presence of the non-specific agonist NECA (100 µM) and antagonist CGS 15943 (100 nM). Channel openings were recorded for at least 20 min in the absence of treatment (ctrl, autocrine channel activity) and for at least other 20 min after drug superfusion. To reduce membrane distortions introduced by seal formation in the cell attached configuration, which could interfere with second messenger effects, single channel activity was measured in different membrane patches before and after drug treatment, immediately after achieving the gigaseal.

In all the cells tested, NECA increased the mean open time and the \(P_o\) of channel openings (Control: mean open time, 7.24 ± 0.58 ms, n = 10; \(P_o\), 0.060 ± 0.014, n = 11; NECA: mean open time, 9.19 ± 0.43 ms; \(P_o\), 0.13 ± 0.017, n = 7; \(p < 0.01\)), whereas the antagonist CGS 15943 significantly reduced the mean open time (5.03 ± 0.26 ms, n = 7; \(p < 0.001\)) without changing the \(P_o\) (0.062 ± 0.016, n = 8; Fig. 4). In all the experiments, no change of ion channel conductance was observed (data not shown).
Fig. 4. Effect of the aspecific ARs modulation on nAChR biophysical properties. A. Effect of ARs modulation of nAChR activity in untreated conditions (Control) and in the presence of NECA 100 µM or CGS 15943 100 nM. The ARs agonist NECA increased the mean open time and the channel $P_o$, whereas the ARs inhibitor CGS decreased the mean open time without affecting the $P_o$ (statistical significance: ** $p < 0.01$ vs control). B. Representative traces of autocrine channels openings in ctrl conditions and in the presence of NECA and CGS.
Discussion

Our data suggest that ARs activity controls nAChR-driven $\left[\text{Ca}^{2+}\right]_i$ transients in differentiating myotubes. In fact, $\left[\text{Ca}^{2+}\right]_i$ imaging revealed that the frequency of $\left[\text{Ca}^{2+}\right]_i$ spiking activity increased in the presence of NECA, while it was blocked in the presence of the antagonist CGS 15943. The inhibitory effect of adenosine deaminase (ADA) on $\left[\text{Ca}^{2+}\right]_i$ spiking activity of twitching myotubes suggests the presence of endogenous adenosine in our conditions and that ARs are possibly tonically active, in agreement with the contraction-dependent release of adenosine reported in literature\textsuperscript{102,103}.

Taking into account what we have observed in the presence of the specific AR antagonists, even if still not conclusive, our results pointed out a prevalent role of $\text{A}_2\text{B}$Rs. $\text{A}_2$Rs were previously proposed to modulate the nAChR channel properties via the intracellular cAMP signalling\textsuperscript{98}. In this study, the authors demonstrated a negative adenosine-mediated regulation of nAChR activity. In contrast, our preliminary results indicate the existence of a potential positive feedback ARs-nAChRs. A possible explanation for this apparent discrepancy could be that Pitchford and coworkers have studied the adenosine effect not under basal conditions, but in the presence of a cholinergic agonist that increased significantly adenosine secretion and thus its endogenous content.

In conclusion, our preliminary results are in favour of the role of adenosine in the regulation of the nAChR-dependent ($\left[\text{Ca}^{2+}\right]_i$) spikes during postnatal myogenesis. Further studies are required to better identify the contribution of each specific AR isoform on the biophysical properties of the nAChR single openings. Moreover, to better characterize the signalling mechanism between ARs-nAChRs, it would be of great interest to study the distribution of AR subtypes on differentiating myotubes and to identify which of the AR isoform co-localises with the nAChRs.
7. The “non-canonical” roles of neural agrin during myogenesis

The heparan sulfate proteoglycan agrin was discovered almost 30 years ago, when McMahan and colleagues observed that components of the synaptic basal lamina carry instructive information necessary to induce pre- and postsynaptic specializations during neuromuscular regeneration\textsuperscript{104,105}. Agrin is synthesized mainly by motor neurons, transported along motor axons and stably incorporated into the basal lamina of the synaptic cleft\textsuperscript{106,107}, where it acts as a crucial organizer of neuromuscular junction. In fact, agrin deficient mutant mice die perinatally, due to respiratory failure as a consequence of a non-proper development of the NMJs throughout the body muscles\textsuperscript{108}. The agrin gene encodes for a protein with more than 2000 amino acids, with a molecular weight of approximately 225 kDa, that can switch to higher values (up to 400-600 kDa). Indeed, the molecule owns numerous sites, like heparan sulfate and condroityn sulfate domains, that can be extensively O- and N-linked glycosylated\textsuperscript{109} (Fig. 8). The transcript of the agrin gene can be differentially spliced to produce different isoforms, each with its function and tissue distribution.

Outside the skeletal muscle, this protein is broadly expressed in several other tissues, for instance in brain\textsuperscript{107}, kidney\textsuperscript{110}, heart\textsuperscript{111} and activated lymphocytes during immune response\textsuperscript{112}. Its distribution indicates a wide range of effects of the molecule, most of which have been poorly investigated to date\textsuperscript{113}. The agrin isoform synthesized and released by motor neurons, referred to as neural agrin, owns a N-terminal SS-NtA domain, through which it stably binds to the basal lamina of skeletal muscle cells. While the N-terminus of agrin is important to determine tissue distribution, the C-terminal half is the recognized “active” portion of the molecule. It comprises four epidermal growth factor-like domains (EG), three laminin G-like domains (LG1, LG2, LG3) and two additional splicing sites, known as A/y and B/z (A and B refer to the chick, y and z to mammals)\textsuperscript{114}. The alternative splicing at this level produces agrin isoforms lacking or containing specific amino acidic inserts. The A/y domain is placed in the LG2 globule and can contain a sequence of 0 or 4 amino acids (Lys-Ser-Arg-Lys)\textsuperscript{114}. Although the physiological role of the A/y insert is still not clear, it seems that it regulates agrin binding to heparin\textsuperscript{115}. The B/z site is located in proximity of the LG3 domain, and it...
can contain sequences of 0, 8, 11 or 19 amino acids. The inclusion of inserts at B/z site (B/z+ form) is of great importance for agrin activity at the NMJ\textsuperscript{116}. This domain is considered the active site of the molecule in triggering the aggregation of AChRs, and for this reason neural agrin is also named as the “active isoform”\textsuperscript{114}.

Fig. 8. Agrin structure. At the N-terminus agrin can either display a SS-NtA domain (present in the neural form) or a transmembrane domain (TM). The NtA is required for binding to laminins, while the TM portion identifies agrin isoforms whose N-terminal is incorporated into the cell membrane. The central region is constituted by a rod-like structure, with several glycosilation and glycosaminoglycan (GAG) attachment sites. The C-terminal part of agrin owns three laminin-like domains (LG,) containing two splice sites, the A/y and the B/z. The four-amino acid insert at splice site A/Y binds to heparin, and the insert at B/z site is important to induce aggregation of AChRs. The first two LG domains are involved in α-dystroglycan binding (modified from [113]).

The key mediator of neural agrin signaling is the muscle-specific receptor tyrosine kinase, MuSK, expressed by the skeletal muscle fiber and concentrated at the post-synaptic region, where it co-localizes with the AChRs\textsuperscript{117} (Fig. 9 A). The receptor is a crucial player in NMJ formation. Knockout experiments demonstrated that the suppression of MuSK expression leads to a phenotype similar to that of agrin-deficient animals\textsuperscript{118}. In addition, cells derived from MuSK\textsuperscript{-/-} mice are incapable of aggregating AChRs in response to agrin\textsuperscript{119}.

MuSK is highly expressed by myotubes and skeletal muscle fibers and co-localizes with the AChRs at the NMJ\textsuperscript{120}. It consists of an extracellular portion, with 4 immunoglobulin-
like domains and a cysteine-rich domain; a transmembrane fragment (TM); an intracellular region with a juxtamembrane tyrosine (Y553), followed by a catalytic domain made up of 17 tyrosine residues\(^{117}\) (Fig. 9 B). MuSK phosphorylation does not occur following a direct agrin binding to the receptor\(^{119,121}\). Its activation is mediated by a co-receptor, belonging to the low-density lipoprotein receptor family, LRP4\(^{122}\), capable of recognizing specifically the neuronal isoform of agrin\(^{121,122}\).

**Fig. 9.** A. The receptor MuSK is expressed by skeletal muscle cells at the postsynaptic membrane of the NMJ, where it co-localizes with the AChRs. B. Schematic representation of MuSK structure: the receptor owns an extracellular N-terminal domain, a transmembrane region (TM) and an intracellular C-terminal domain that displays the kinase activity (modified from [\(^{117}\)]).

Binding of agrin to LRP4 stimulates MuSK dimerization and the consequent phosphorylation of specific intracellular sites. MuSK downstream pathway, leading to AChR clustering, constitutes a complex network of crosstalking signaling molecules\(^{123,124}\) (Fig. 10).
Fig. 10. MuSK downstream pathway regulating the nAChR clustering. Agrin triggers MuSK dimerization with consequent phosphorylation of the intracellular tyrosine residues. Several effector proteins, such as Dok7 and Abl kinases, reciprocally interact with the receptor MuSK and contribute to the generation of the downstream signaling pathway. Actin remodeling drives AChR trafficking, insertion and immobilization and the aggregation of the AChR is stabilized by rapsyn (modified from [124]).

In recent years, non-canonical roles of agrin have come to the fore, expanding its range of effects during myogenesis. These non canonical effects include remodelling of the muscle cell surface and cytoskeletal elements, maturation of the excitation-contraction coupling mechanism, modulation of the electrical properties and contractile function.

Considering that in mammals there seems to be a peculiar concentration of satellite cells in the proximity of the nerve terminals, the mammalian myogenic precursors may be physiologically exposed to nerve-derived trophic factors like agrin.

In this light we explored the effect of neural agrin on the control of functional properties of satellite cells. The research activity was aimed at investigating if neural agrin controlled the secretion of IL-6 and the proliferative capacity of the myogenic precursors. Our results revealed that IL-6 extracellular levels are increased following exposure to agrin (7.1. Appendix 3 – Published results) and that agrin could enhanced the proliferative capacity of human satellite cells (7.2. Appendix 4 – Preliminary results).
APPENDIX 3 – PUBLISHED RESULTS
Non-Synaptic Roles of Acetylcholinesterase and Agrin

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Abstract Proteins in living organisms have names that are usually derived from their function in the biochemical system their discoverer was investigating. Typical examples are acetylcholinesterase and agrin; however, for both of these, various other functions that are not related to the cholinergic system have been revealed. Our investigations have been focused on the alternative roles of acetylcholinesterase and agrin in the processes of muscle development and regeneration. Previously, we described a role for agrin in the development of excitability in muscle contraction. In this study, we report the effects of agrin on secretion of interleukin 6 in developing human muscle. At the myoblast stage, agrin increases interleukin 6 secretion. This effect seems to be general as it was observed in all of the cell models analysed (human, mouse, cell lines). After fusion of myoblasts into myotubes, the effects of agrin are no longer evident, although agrin has further effects at the innervation stage, at least in in vitro innervated human muscle. These effects of agrin are another demonstration of its non-synaptic roles that are apparently developmental-stage specific. Our data support the view that acetylcholinesterase and agrin participate in various processes during development of skeletal muscle.

Keywords Agrin · Acetylcholinesterase · Muscle cells · Interleukin 6 · Neuromuscular junction

Introduction

Among the many proteins involved in development, assembly and maintenance of neuromuscular junctions (NMJs) (Burden 2002), acetylcholinesterase (AChE) and neural agrin have attracted great attention. Acetylcholinesterase (EC 3.1.1.7) is anchored to the basal lamina of the NMJ, and it is responsible for the termination of synaptic transmission (Rotundo 2003; Massoulié and Millard 2009). Neural agrin is a trophic factor that is released by nerve terminals (Nitin et al. 1987), and it has been identified as one of the crucial controllers of the postsynaptic apparatus (McMahan 1990). This synaptic activity of neural agrin is based on its binding to the Lrp4/MuSK receptor complex (Kim et al. 2008; Zhang et al. 2008; Ghazanfari et al. 2011; Zong and Jin 2013) and the related signalling cascade that controls growth and stabilisation of acetylcholine receptor (AChR) clusters and expression of other important components of the postsynaptic membrane. Although they have completely different synaptic roles, AChE and neural agrin share numerous common features (Mis et al. 2013). They are both expressed in both the neural and muscle tissue of the NMJ, they are both expressed by a single gene, followed by alternative splicing, and they both have additional biological activities that are not directly linked with the cholinergic system and synaptic transmission. Indeed, it was shown that AChE controls cell adhesion, neurite growth and network formation, embryonic development in the central nervous system (Paraoanu et al. 2006), amyloid fibre assembly in brain (Inestrosa et al. 2008), haematopoiesis (Soreq et al. 1994), stress responses (Meshorer and Soreq 2006), apoptosis and inflammation (Pegan et al. 2010; Tsim and Soreq 2012; Zhang and Greenberg 2012). The non-synaptic effects of neural agrin include remodelling of the muscle cell surface (Uhlm et al. 2001) and cytoskeletal elements (Bezakova et al. 2001), maturation of the excitation–contraction coupling mechanism (Bandi et al. 2008),...
modulation of the electrical properties (Jurdana et al. 2009) and contractile function of developing skeletal muscle cells (Bian and Bursac 2012).

In light of its non-synaptic roles, we have reported on the influence of agrin in the development of skeletal muscle (Mis et al. 2013). Here, we report the results of our recent investigations that have focused on the role of neural agrin in the control of cytokine signalling at the skeletal muscle level. Skeletal muscle cells express and secrete interleukin 6 (IL-6) and other cytokines, both constitutively (Prelovsek et al. 2006; reviewed in Pedersen and Febbraio 2008) and in response to various factors (Puppa et al. 2012; Podbregar et al. 2013). It is generally accepted that secretion of IL-6 is a major cytokine response in skeletal muscle to various environmental stimuli, and that autocrine and paracrine effects of secreted IL-6 influence muscle development (Pedersen and Febbraio 2008). Using an in vitro approach, we have here explored the effects of neural agrin on IL-6 secretion at different developmental stages in cultured skeletal muscle cells: in the mouse C2C12 muscle cell line, and in primary mouse and human muscle cells.

**Material and Methods**

The experiments on human muscle cells were approved by the National Medical Ethics Committee of the Ministry of Health of the Republic of Slovenia (permit numbers 63/01/99 and 71/05/12) and were conducted in accordance with the Declaration of Helsinki and Good Laboratory Practice regulations. Experiments on mouse tissues were planned to use limited number of animals and to minimise their suffering, and the procedures were approved by the Veterinary Administration of the Ministry of Agriculture and the Environment of the Republic of Slovenia (permit number 323-02-74/00).

**Agrin Treatment**

Recombinant full-length chick neural agrin (cAgrin7A4B8; kindly provided by G. Fumagalli, University of Verona, Verona, Italy) was purified from conditioned media of stably transfected HEK 293 cells using mono-Q-Sepharose fast-flow beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA), as described previously (Bandi et al. 2008). In all of the cell cultures, the agrin treatments were performed by replacing the culture medium with medium supplemented with 1 nM full-length neural agrin for 24 h, except in in vitro innervated muscle cell cultures, where the cells were treated with agrin for 3 days. The supernatants were collected after these 24-h or 3-day treatments and frozen at −80 °C until determination of IL-6 concentration. The cells were fixed and stained for nuclei counting; using Hoechst 333424 (see below).

**IL-6 Detection**

The levels of IL-6 secreted in the supernatants collected from the cell cultures were measured using enzyme-linked immunoabsorbent assay (ELISA) kits (Endogen, Rockford, USA), according to the manufacturer instructions, and as described previously (Rezonja et al. 2013). To reach comparability of released IL-6 from different cell cultures and between myoblasts and myotubes, the amounts of IL-6 were calculated per 10⁶ nuclei, except in innervated muscle cultures, where the total amount of IL-6 released into the culture medium was measured and used, rather than calculated per number of nuclei. The numbers of the nuclei were determined by Hoechst 333424 staining. Briefly, the cells were fixed with 4% paraformaldehyde and incubated with 1 μg/ml Hoechst 333424 for 5 min at room temperature. The counting of the nuclei was carried out for at least five optical fields per well, and at least three wells were analysed for each experimental point.

**Calcium Imaging**

Calcium imaging was used to check the presence of the skeletal-type excitation–contraction coupling mechanism, assessed as occurrence of an intracellular Ca²⁺ transient when myotubes were depolarized with 40 mM KCl, in a Ca²⁺-free solution (for further details, see Bandi et al. 2008). Briefly, myotubes were loaded with the fluorescent Ca²⁺ indicator fura-2 pentacetoxymethylster (5 μM) at room temperature (30 min, in the dark). During Ca²⁺ measurements the temperature was kept at 37 °C and cells were excited alternately at 340 and 380 nm. Fluorescence images were acquired by a CCD camera (SensiCam; PCO Computer Optics, Kelheim, Germany) at rate of 4 images/s. Calcium transients were measured as variation in the mean value of the fluorescence intensity in ratio images (340/380). The fluorescence at rest was normalised to value 1. Variations in the intracellular concentration of the ion were expressed as increase in fluorescent signal relative to the fluorescence at rest.

**Primary Human Muscle Cell Cultures**

Primary human muscle cell cultures were derived from satellite cells isolated from muscle tissue obtained from orthopaedic surgery patients who had been previously diagnosed to be without neuromuscular diseases. To release the satellite cells, the human muscle tissue was separated from the connective tissue, dissected into 0.5-mm to 1.0-mm pieces, and trypsinised. The satellite cells were then grown to clonal density in 100-mm Petri dishes in advanced Minimal Essential Medium (Invitrogen Gibco, Paisley, UK) containing 10% (v/v) foetal bovine serum (FBS), at saturating humidity in a mixture of 5% CO₂ and air, at 37 °C. The myoblast colonies were further selected from the fibroblast contaminants by morphological criteria.
Myoblast cultures prepared from different donors were checked prior their use for myogenicity by desmin counting, and expanded in the advanced Minimal Essential Medium with 10 % FBS, as described previously (Pegan et al. 2010; Rezonja et al. 2013). The myoblasts were plated in six-well dishes and cultured for 3 days before exposure to neural agrin.

To induce differentiation into myotubes, human myoblast cultures plated in six-well dishes were shifted to differentiation medium after 3 days (advanced Minimal Essential Medium supplemented with 2 % FBS). After myoblast fusion, the myotube cultures were maintained in differentiation medium for an additional 10 days before neural agrin exposure.

To obtain innervated human myotubes, the myoblasts were plated on glass coverslips coated with a 1:2 mixture of 1.5 % gelatin (Sigma-Aldrich, St. Louis, MO, USA) and human serum, in 35-mm six-well dishes. Subsequently, the myoblast cultures were grown in F14 medium (Invitrogen) supplemented with 10 % FBS, 50 ng/ml fibroblast growth factor, 10 ng/ml epidermal growth factor, and 10 μg/ml insulin (all from Sigma-Aldrich). After the myotubes formed from the myoblasts, up to four segments of dissected spinal cord explants with preserved anatomical connections to dorsal root ganglia and preserved meninges were placed onto the myotube monolayers. To isolate the embryonic spinal cords, timed pregnant Wistar rats were sacrificed at 14 days from gestation in a CO2 chamber. Over the following 7–10 days, these explant cocultures were cultivated in F14 medium with 5 % (v/v) FBS and 10 μg/ml insulin, with this medium replaced with fresh medium every 2 to 3 days. Functional innervation was regularly monitored microscopically, as described in detail previously (Mars et al. 2001; Rezonja et al. 2013). Neural agrin exposure was carried out when functional innervation (i.e., contraction-positive co-cultured explants) reached a steady-state.

Primary Mouse Muscle Cells

Primary mouse myoblasts were established from satellite cells as described in more detail elsewhere (Irntchinev et al. 1997). The mouse myoblasts were maintained in HAM’S F-10 medium (ICN Biomedicals, Costa Mesa, CA, USA) containing 20 % foetal calf serum (PAA Laboratories, Linz, Austria), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (all Sigma), for 1 day, before exposure to neural agrin.

To obtain mouse myotubes, 1 day after the myoblast plating, the medium was replaced with Dulbecco’s modified Eagle’s medium (ICN Biomedicals) supplemented with 2 % horse serum (ICN Biomedicals), and L-glutamine, penicillin and streptomycin, as above. The cells were maintained at 37 °C in a mixture of 5 % CO2 and air. The differentiation medium was renewed every 2 days. After 7 days in differentiation medium, the mouse myotubes were treated with neural agrin.

C2C12 Muscle Cell Line

The mouse C2C12 muscle cell line was from Developmental Studies Hybridoma Bank (Tacoma, Washington, USA). The C2C12 myoblasts and myotube cultures were prepared and grown using standard protocols, as described previously (Gajsek et al. 2006). Treatment with neural agrin was carried out with the same timing as that described for the primary mouse myoblast and myotube cultures.

Statistical Analysis

All of the experimental data are expressed as means±S.D., and were obtained from three independent experiments with multiple repeats for each experimental condition. Significance was determined using Student’s t tests, with p < 0.05 considered statistically significant. Univariate two-way analysis of variance (ANOVA) was used to test for differences in IL-6 secretion in the control and agrin-treated cell cultures. Statistical analysis was performed using IBM SPPS Statistics 20.0 for Windows (IBM Corp., Armonk, NY, USA).

Results

The Effects of Neural Agrin on IL-6 Secretion in Primary Human Muscle Cell Cultures

The effects of neural agrin (1 nM, 24 h exposure) on the secretion of the pro-inflammatory cytokine IL-6 was followed at three differentiation stages of primary human muscle cell cultures: myoblasts, myotubes, and in vitro innervated myotubes. Human muscle cells aneurally grown or innervated appeared highly differentiated morphologically (presence of long multinucleated myotubes) and physiologically (presence of skeletal-type excitation–contraction coupling mechanism assessed by Ca2+ transients after depolarization in Ca2+ free conditions) (Fig. 1a, b).

A significant 1.24-fold increase in constitutive IL-6 secretion was detected in agrin-treated myoblasts when compared with the control, untreated myoblasts (7.88±0.89 vs. 6.38±0.79 ng IL-6/105 nuclei; p < 0.05). After myoblast fusion into myotubes, the IL-6 levels increased under both control conditions and after exposure to neural agrin. The level of IL-6 released from the myotubes was significantly higher than in the myoblasts under control conditions (1.59-fold; p < 0.05). After exposure of human myotubes to neural agrin, there was a trend to an increase in the IL-6 content (1.06-fold), although this effect of agrin was not statistically significant (10.77±3.23 vs. 10.18±3.03 ng IL-6/105 nuclei) (Fig. 2). The IL-6 secretion was also evaluated in functionally innervated human myotubes. At day 11 of culture with spinal cord explants, the myotubes were treated with 1 nM agrin for 3 days. In the
Fig. 1 Differentiation in the cell models analysed. Primary human (a, b) and mouse (c) muscle cell cultures, C2C12 muscle cell (d) are shown as representative phase-contrast micrographs of differentiated myotubes with corresponding Ca$^{2+}$ transients elicited by depolarization in Ca$^{2+}$-free conditions, confirming the establishment of the functional skeletal-type excitation-contraction coupling mechanism. Scale bars in phase-contrast micrographs, 30 μm.

control co-cultures, the mean level of constitutive IL-6 secretion was 24.64±11.47 μg/ml. In the agrin-treated co-cultures, the IL-6 concentration was significantly higher, at 1.75-fold the control (43.04±7.14 μg/ml; p < 0.05) (Fig. 3).

The Effects of Agrin on IL-6 Secretion in Primary Cell Cultures and C2C12 Mouse Muscle Cells

Primary and C2C12 mouse myoblasts and myotubes were treated with neural agrin at the same concentration as for the human muscle cells (1 nM). As in human primary muscle cell model,

primary mouse and C2C12 myotubes appeared well differentiated by morphological and physiological criteria (Fig. 1c, d). Similar to our observations with the human muscle model, after agrin treatment, there were significant increases in IL-6 secretion in the primary muscle cells as well as in the C2C12 myotubes. These increases with respect to their corresponding controls were 1.54-fold in agrin-treated primary myoblasts (9.98±1.71 vs. 6.48±0.18 ng IL-6/10^5 nuclei; p < 0.05), and 1.16-fold in agrin-treated C2C12 myoblasts (1.81±0.11 vs. 1.56±0.09 ng IL-6/10^5 nuclei; p < 0.05) (Fig. 4). In the myotube models of both the primary cultures and the C2C12 cells, the exposure to agrin did not significantly affect the constitutive secretion of IL-6 (primary: 13.04±1.80 vs. 9.88±0.80 ng IL-6/10^5 nuclei; C2C12: 2.02±0.23 vs. 1.81±0.14 ng IL-6/10^5 nuclei), although there were higher levels of constitutive IL-6 secretion in these myotubes when compared to the myoblasts (Fig. 4).

Fig. 2 Secretion of IL-6 from control and agrin-treated human myoblasts and myotubes. The primary human myoblasts and myotubes were treated with 1 nM agrin for 24 h. The medium was then removed and IL-6 secretion was estimated by ELISA, with the amounts of IL-6 released calculated per 10^5 nuclei. Data are means±SD, n indicates total number of repeats analysed. *p < 0.05 vs. control.

Fig. 3 Secretion of IL-6 from the innervated control and agrin-treated human muscle cell cultures. In the human myotubes co-cultured with rat spinal cord explants, the treatment with neural agrin (1 nM, 3 days) increased the constitutive secretion of IL-6. Data are means±SD, n indicates total number of repeats analysed. *p < 0.05 vs. control.
Discussion

Here, we have demonstrated that at the myoblast stage of muscle differentiation, neural agrin increases IL-6 secretion. This effect was observed in all of the cell models analysed here (human, mouse, cell lines). After the fusion of these myoblasts into myotubes, this effect of neural agrin was not evident any more, although neural agrin increased IL-6 secretion again at the innervation stage, at least in this model of innervated human muscle cells. This effect of agrin has not been reported before, and it is another demonstration of the non-synaptic roles of agrin, which appear to be diverse and developmental-stage specific.

Interleukin 6 is a major muscle-derived cytokine that is expressed and secreted from adult skeletal muscle under resting conditions (Pedersen and Febbraio 2008). During exercise and in regenerating muscle, IL-6 secretion is markedly increased (Kurek et al. 1996; Kami and Senba 1998). Additionally, muscle progenitor cells, myoblasts and myotubes, constitutively express and secrete IL-6 (De Rossi et al. 2000; Prelovsek et al. 2006). The best known role for IL-6 is its modulation of immune cell responses and regulation of energy metabolism (Al-Khalili et al. 2006). Here, the IL-6 expression and secretion in developing and regenerating muscle cells indicates its additional role as a promoter of myoblast proliferation, myotube formation, and induction of muscle hypertrophy (Austin et al. 1992; Serrano et al. 2008).

The regulation of IL-6 expression and secretion from muscle precursor cells appears to be particularly complex and is not yet completely understood, although it is known to be affected by different factors and conditions. Tumour necrosis factor (TNF)-α, IL-1, and lipopolysaccharide that are produced in activated immune cells and bacteria, and also endogenously, are known to modulate IL-6 secretion from muscle cells (De Rossi et al. 2000; Prelovsek et al. 2006; Podbregar et al. 2013). In addition to these factors, exposure of muscle cells to glucocorticoids and hypoxia modulates IL-6 secretion (Prelovsek et al. 2006; Pirkmajer et al. 2010). Our data in the present study demonstrate that this cytokine signalling can also be controlled by neurotrophic factors. Thus, this enhancing effect of neural agrin on IL-6 secretion in human and mouse primary muscle cell cultures, and also in the mouse C2C12 muscle cell line, suggests that the control by this neurotrophic factor is not a peculiarity of the human model, which is well known as being more strictly dependent on neural input (Gajsek et al. 2006; Tanaka et al. 2000).

In the present study, 1 nM neural agrin was used because of previous reports showing that at such concentration the neurotrophic factor has non-synaptic action (Bandi et al. 2008; Mis et al. 2013). The effects here reported are to some extent in agreement with the mechanisms underlying the role of agrin in the formation of NMJs. These effects include the release of agrin from the motor nerve endings, and its binding to and activation of the MuSK tyrosine kinase receptor in the target muscle cell (Glass et al. 1996). As a synaptic organiser, agrin is known to induce AChR aggregation, although it also has marked effects on gene expression in the synaptic nuclei, which are mediated through a complex network of signalling pathways (Bezakova and Ruegg 2003). The signalling pathway that is most closely linked to IL-6 expression and secretion is STAT3 signalling (Heinrich et al. 1998). However, at present, there is no direct evidence that supports a link between agrin activation of the MuSK signalling pathway and activation of the STAT3 signalling pathway.

Interestingly, the effects of agrin on IL-6 secretion appear to be dependent on the stage of muscle cell differentiation, with more pronounced effects in myoblasts and in innervated muscle than in myotubes, where there was no significant increase in IL-6 secretion upon treatment with agrin. This observation is in agreement with previous reports where TNF-α, lipopolysaccharide- and glucocorticoid-dependent IL-6 secretion was more pronounced in myoblasts than in myotubes (Prelovsek et al. 2006). The different effects in myoblasts, myotubes and innervated muscle cells might be due to the well-known biological and phenotypical differences between proliferating myoblasts, differentiated multinucleated myotubes, and innervated and contracting myotubes, in terms of protein expression and responses to different trophic and extrinsic factors (Hauschka and Emerson 2004; Cicilliot and Schiaffino 2010). In addition, this differentiation-dependent agrin sensitivity might be due to differences in the ‘agrin receptor’ complex in different cell types (Glass et al. 1996; Bezakova and Ruegg 2003).

The non-synaptic role of agrin described here is in agreement with the numerous other non-synaptic roles of agrin that have been reported to date. Thus, agrin is an example of a protein where the alternative splicing of a single gene can produce isoforms with several different activities. A close
counterpart here is AChE, which is another protein that is detected at the level of the NMJ that has more recently discovered functions that are not related to the NMJ and the cholinergic system (Mis et al. 2013). Different from their nonsynaptic (non-canonical) roles which seem to be separated, AChE and agrin are interconnected in the processes of skeletal muscle development and in their synaptic activities. AChE is involved in myoblast apoptosis (Pegan et al. 2010), while agrin is involved in control of excitation–contraction coupling mechanism (Bandi et al. 2008) and contractile apparatus maturation (Mis et al. 2013). Treatment with agrin results not only in aggregation of existing AChR into clusters (Tsim and Barnard 2002) but also in a rapid translocation of receptors to the AChE clusters (Rotundo et al. 2005). Synaptic transmission organized through synaptogenetic actions of agrin induces AChE appearance at the NMJ (Sanes et al. 1998). Accumulation of AChE in dense patches which is a part of NMJ formation (Grubic et al. 1995; Siow et al. 2002; De Jaco et al. 2005; Camp et al. 2008; Tsim et al. 2010) is also induced by agrin. However, synaptogenetic actions of agrin can be modulated by AChE as shown by Choi et al. (1998) where over-expression of AChE promotes postsynaptic specialization through stimulation of agrin expression.

In conclusion, the present study reveals another non-canonical function of neural agrin, which supports the view that as well as having a role in NMJ assembly and cholinergic transmission, this neurotrophic factor participates in various other processes that take place during differentiation of skeletal muscle. Our observations provide new insights into the involvement of neural agrin, and more in general of neurotrophic factors, in the control of skeletal muscle plasticity and regeneration.

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References

Neural agrin positively affects the proliferation of old donor-derived human myoblasts

Introduction

As already mentioned in the general introduction, aging of the skeletal muscle is characterized by a severe reduction in tissue mass, strength and endurance. The impaired regenerative response of satellite cells during aging is widely accepted but the molecular causes underlying this phenomenon are still partially unclear. Among the possible reasons, changes in the composition of the systemic and/or local environment may be responsible for satellite cell diminished regenerative ability. The influence of the external milieu was demonstrated by transplanting experiments of old muscles into young animals, where satellite cell regeneration was successfully restored.74

Beside local chemical signals released by satellite cells, myofibers, and neighbouring non-muscle cells, nerve-derived factors may have a an important impact in regulating satellite cells behaviour and their level are possibly reduced during aging. In fact, aging of the skeletal muscle is characterized by an extensive loss of motor neuron contacts. Even if the contribution of nerve-derived factors has not been extensively characterised, it is known that denervation contributes to the progression of sarcopenia, leading to abortive myogenesis129 and loss of muscle mass68,130. The magnitude of denervation is much higher in human muscle, as compared to rat muscles, suggesting that humans are more susceptible to age-related denervation and atrophy131,132. Among nerved-derived trophic factors whose levels are possibly reduced as a consequence of age-related denervation, neural agrin is a promising candidate. Very recently, an elevated agrin degradation has been demonstrated in a large subpopulation of sarcopenic human patients, suggesting a role for agrin in the pathogenesis of sarcopenia133.

In the light of this evidence, and of others indicating agrin as a key factor controlling proliferation/survival134, the specific aim of the present research activity was to explore the possible role of neural agrin as controller of human satellite cell proliferation.
Methods

Isolation and purification of human skeletal myoblasts

Experiments were approved by the Ethics Commission at the Ministry of Health of the Republic of Slovenia or by the Ethics Commission of the Medical Faculty of the University of Bonn. Primary human myoblast cultures were prepared from skeletal muscle tissue discarded during routine orthopaedic operations. Donors or their parents (legal representatives) signed an informed consent agreeing that the skeletal muscle tissue could be used for experimental purposes. None of the donors had neuromuscular disease. Cell cultures were prepared as described elsewhere in more detail\textsuperscript{126,135,136}. Briefly, skeletal muscle tissue was cleaned of visible connective and adipose tissue and then cut into small (0.5-1 mm) pieces under the microscope. This was followed by trypsinization (0.15%; 37 °C for 30-45 min) in Earle’s Balanced Salt Solution (EBSS) to digest basal lamina and release the satellite cells. The cell suspension was then centrifuged (1000 rpm; for 5 min), pellet was resuspended and cells were plated into cell culture dishes. Primary human skeletal muscle cells were grown, at 37 °C in humidified atmosphere (95% air/5% CO\textsubscript{2}), in Advanced MEM, supplemented with 10% (v/v) FBS, 0.3% (v/v) fungizone and 0.15% (v/v) gentamicin. Three-week old primary cultures were trypsinized and then purified using MACS CD56 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in order to separate the myoblasts from other cell types (mainly fibroblasts) contaminating the primary culture. After purification and desmin staining (see below), myoblasts were used for experiments or frozen in liquid nitrogen until further use.

Old and young donor-derived myoblasts

Primary myoblast cultures were prepared from skeletal muscle samples collected from two groups of donors. The first group consisted of donors whose age ranged from 5 to 15 years (young myoblasts), the second of donors ranging from 50 to 80 years of age (old myoblasts). Before their use, the percentage of myogenic cells was assessed by desmin staining. Cell cultures with a desmin counting higher than 75% were selected for the experiments here described.
C2C12 cell culture

Mouse C2C12 myoblasts (Developmental Studies Hybridoma Bank, Tacoma, Washington) were cultivated in proliferative conditions, using Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 20% (v/v) FBS, 4 mM L-glutamine, 100 IU mL⁻¹ penicillin and 100 μg mL⁻¹ streptomycin. Each time that cells reached 75% confluence, they were trypsinized and replated. Cells were maintained at 37 °C in an atmosphere humified 100% (95% air/5% CO₂).

Growth curves and cumulative number of population doublings

The proliferative capacity of myoblasts from different donors was assessed by estimating their growth curves and cumulative number of population doublings (CPD) over the entire course of the in vitro lifespan. The two parameters were evaluated in growth medium (GM) or in GM supplemented with 1 nM full-length neural agrin. GM consisted of Ham’s F10 supplemented with FBS (20%; Euroclone, Pero, Italy), 1 mM L-glutamine, 100 units mL⁻¹ penicillin and 100 μg mL⁻¹ streptomycin. In the two experimental paradigms, human myoblasts were plated at the same density (14 cells/mm²). Control and agrin-treated myoblasts were both expanded until the sub-confluent state and counted using a Coulter counter. At each cell passage, the mean population doubling (MPD) was calculated as MPD = LogN/Log2, where N is the number of cells harvested divided by the number of cells seeded. For each experimental point, at least three different dishes were analysed; for each cell sample, cell number was determined in duplicate. Cell cultures were considered at the end of their in vitro lifespan (senescence), when their number remained constant during two consecutive sub-cultures. Myogenicity was routinely checked by immunocytochemistry for desmin (see below). GM was changed every 3 days.

BrdU proliferation assay

Bromodeoxyuridine (BrdU) Cell Proliferation Assay Kit was used to estimate the rate of myoblast proliferation. Human myoblasts were plated in 96-well plates in GM at the density of 50 cells/mm². The day after seeding, myoblasts were exposed to neural or mini-agrin and/or imatinib or PD185352 for 24, 48 or 72 h. Myoblasts were then
incubated with BrdU for additional 16 h. Samples were prepared for BrdU absorbance measurement according to the manufacturer’s protocol. Absorbance was measured at dual wavelengths (450 and 550 nm) using Victor 3 plate reader (PerkinElmer, Shelton, Connecticut, USA). Results were expressed as the difference between absorbance at 450 nm and 550 nm or as percent-control. Each independent experiment was done in at least 4-8 replicates for each treatment.

**Western Blot**

Human myoblasts were washed with ice-cold PBS and then harvested with lysis buffer (1% (v/v) Protease Inhibitor Cocktail, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 0.5 mM Na₂VO₄, 1% (v/v) Triton X-100, 1% (v/v) glycerol, 20 mM Tris, 10 mM NaF, 1 mM EDTA, and 1 mM phenylmethanesulfonfyl fluoride). Lysates were centrifuged at 4 °C (12000 g, 15 min) and supernatants were collected. Total protein concentration was measured with Bicinchoninic Acid (BCA) Protein Assay Kit. For SDS-PAGE, collected supernatants were diluted to the same final protein concentration and incubated in Laemmli buffer (62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (w/v) glycerol, 0.002% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol) for 20 min at 56 °C. Protein samples were separated on 4-12% Bis-Tris gel and transferred to PVDF membrane using Criterion system (Bio-Rad, Hercules, California, USA). To assess sample loading and the efficiency of the transfer, membranes were stained with 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid. Membranes were blocked in 7.5% (w/v) non-fat milk in TBS-T (10 mM Tris, 137 mM NaCl, 0.02% (v/v) Tween-20, pH 7.6) and subsequently probed with primary antibodies against MuSK or total Abl overnight at 4 °C. GAPDH was used as loading control. Following overnight incubation, membranes were incubated with the appropriate HRP-conjugated secondary antibody. Immunoreactive proteins were visualized using enhanced chemiluminescence and analysed with Chemigenius Bio-imaging System (Syngen, Cambridge, UK).

**Myoblast fusion efficiency**

To estimate the efficiency of myotube formation, fusion index and the mean number of nuclei/myotube were determined. To trigger cell differentiation and fusion into myotubes, myoblasts were plated on matrigel-coated coverslips at 150 cells/mm² in GM
and, three days after plating, GM was substituted with differentiation medium (DM: DMEM supplemented with 2% FBS, 100 μg mL\(^{-1}\) apo-transferrin, 10 μg mL\(^{-1}\) insulin, 4 mM L-glutamine, 100 units mL\(^{-1}\) penicillin and 100 μg mL\(^{-1}\) streptomycin). Fusion index and the mean number of nuclei/myotube were quantified on the 7-12\(^{th}\) day of differentiation (i.e. days in DM). These parameters were determined in five randomly chosen optical fields per coverslip. Fusion index was expressed as the number of nuclei in myotubes divided by the total number of nuclei. Myotubes were defined as cells having more than two nuclei.

**Ca\(^{2+}\) imaging in cultured myotubes**

Ca\(^{2+}\) imaging experiments were carried out to check the presence of the skeletal-type excitation-contraction coupling mechanism, assessed as the cell capability to generate an intracellular Ca\(^{2+}\) transient when depolarized (60 mM KCl) in Ca\(^{2+}\)-free solution (140 mM NaCl, 2.8 mM KCl, 2 mM EGTA, 5 mM MgCl\(_2\), 10 mM glucose, 10 mM Hapes; pH 7.3). To this purpose, cells were seeded and differentiated (7-12 days) on glass coverslips coated with matrigel. For Ca\(^{2+}\) measurements see the detailed protocol described by Sciancalepore and collaborator in Appendix 3\(^9\). For each experimental point at least 6 coverslips were analysed. For each coverslip, a random field with 3-10 myotubes was chosen.

**Desmin staining**

Desmin is an intermediate filament, which is widely used as a marker for cells of myogenic lineage\(^{160}\). For desmin staining assessment, human cells were plated onto matrigel-coated glass coverslips. Before staining, cells were fixed in 4% (w/v) paraformaldehyde, permeabilized with 0.5% Triton X-100 (5 min, room temperature). Then, they were incubated with primary anti-desmin antibody (1:50 in PBS; overnight at 4 °C) and with rhodamine-conjugated secondary antibody (1:200 in PBS; 1 h at room temperature). Cells were also stained for nuclei with Hoechst-33258 (1 μg mL\(^{-1}\)). Images were taken with a fluorescent microscope (Nikon Eclipse E800, Tokyo, Japan.) at 590 nm for desmin and at 461 nm for Hoechst-stained nuclei. The percentage of myogenic cells was estimated as the number of desmin-positive cells divided by the number of Hoechst-
stained nuclei. At least five randomly chosen optical fields in three different coverslips were determined.

**Recombinant neural agrin purification**

Recombinant full-length chick neural agrin (cAgrin\textsubscript{748B8}) was purified from the conditioned media of stably transected HEK-293 cells (kindly provided by G. Fumagalli, University of Verona, Verona, Italy) using mono Q-Sepharose fast flow beads (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA), as previously described\textsuperscript{126,137}.

**Chemicals, reagents and materials**

Cell culture media and reagents were from Invitrogen (Life Technologies, Paisley, UK) and cell culture materials were from Greiner (Greiner Bio-One GmbH, Germany), unless otherwise specified. Matrigel was from BD Biosciences (Bedford, Massachusetts, USA). BrdU Proliferation Assay Kit was obtained from Calbiochem (KGaA, Darmstadt, Germany). MES running buffer, 4-12% bis-Tris gels were obtained from Bio-Rad (Hercules, California, USA). Reagents for enhanced chemiluminescence (ECL) and BCA Protein Assay kit were from Thermo-Scientific Pierce (Rockford, Illinois, USA). Protein molecular weight marker (full range rainbow marker) was purchased from GE Healthcare (Uppsala, Sweden). PVDF membrane was from Millipore (Billerica, Massachusetts, USA). Agfa X-Ray films (Agfa, Zagreb, Croatia) were developed using Curix 60 developing machine (AGFA HealthCare, Greenville, South Carolina, USA). RNeasy Mini Plus kit was from Qiagen (Hilden, Germany).

Recombinant C-terminal fragment of rat agrin (550-AG; mini-agrin) was purchased from R&D Systems (Minneapolis, MN, USA). Abl kinase inhibitor imatinib (STI571; Gleevec\textsuperscript{®}) was kindly provided by Novartis (Varese, Italy) and MEK1/2 inhibitor PD185352 was from Sigma-Aldrich.

**Antibodies**

Antibody against N-terminal of human MuSK was from R&D Systems (Minneapolis, Minnesota, USA). Antibodies against total Abl and GAPDH were from Cell Signaling
Technology (Beverly, Massachusetts, USA). Anti-rabbit and anti-goat horseradish peroxidase (HRP)-conjugated secondary antibodies were from Bio-Rad (Hercules, California, USA) and Thermo Scientific Pierce (Rockford, Illinois, USA), respectively. Goat anti-desmin antibody, used for cytochemistry, was from DakoCytomation (Glostrup, Denmark). Rhodamine-conjugated secondary antibody was from Jackson ImmunoResearch Laboratories Inc. (West Grove, Pennsylvania, USA).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 4.0 Software. ANOVA, followed by Bonferroni’s post hoc test, were used to evaluate the differences among several groups. Unpaired Student t-test was used when only two groups were compared. Results are expressed as means ± SEM. Statistical significance was established at $p < 0.05$. 
Results

Neural agrin increases the cumulative number of population doublings of myoblasts derived from old donors

Growth curves and cumulative number of population doublings (CPD) were evaluated culturing human myoblasts (HM) in presence or absence of 1 nM recombinant full-length neural agrin, until they reached senescence (see Experimental procedures). Experiments were performed on cells isolated from three old donors (“old myoblasts”) and two young donors (“young myoblasts”). Two growth curves representative of old and young myoblasts are shown in Fig. 1A.

Fig. 1. Neural agrin promotes proliferation and stimulates BrdU incorporation of human myoblasts derived from old donors. A. The two graphs show two representative growth curves for old and young group of myoblasts, in the presence of 1 nM neural agrin or in control conditions. In old myoblasts, neural agrin increased the cumulative number of population doublings by ~ 30% (see also Table 1), while the neurotrophic factor did not affect the growth curve in the young. Results are means ± SEM. Statistical differences: **p < 0.01, ***p < 0.001 vs Control. B. The graph shows BrdU absorbance values in control condition and after 24-h treatment with 1 nM neural agrin in three old and three young myoblast populations. Results are mean absorbance values ± SEM. Statistical significance: *p < 0.05, **p < 0.01 vs Control.
Notably, 1 nM neural agrin elevated the CPD of old myoblasts already after 2-4 replicative cell cycles and its effect was maintained throughout the *in vitro* lifespan, including the plateau phase. In old and young cell populations, the desmin counting was regularly carried out from the early up to the late passages, when cells approached the cell cycle arrest and ceased to divide. The fraction of desmin-positive cells remained unaltered in agrin-treated cells as well as in control counterparts, indicating that cell myogenicity was not decreased during agrin treatment (data not shown).

Overall, agrin elevated the cumulative number of cell doublings (CPD) of myoblasts from old donors by ~15 – 45% (Table 1). Conversely, the CPD of myoblasts from young donors remained unaltered even during exposure to lower (100 pM) or higher (50 nM) agrin concentrations (data not shown), excluding the possibility of a dose-dependent effect.

<table>
<thead>
<tr>
<th>Age of donor (years)</th>
<th>Control</th>
<th>1 nM neural agrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>9.95 ± 0.32</td>
<td><strong>13.07 ± 0.14</strong>*</td>
</tr>
<tr>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>4.24 ± 0.09</td>
<td><strong>4.83 ± 0.08</strong></td>
</tr>
<tr>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>1.63 ± 0.02</td>
<td><strong>2.38 ± 0.09</strong>*</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** Age-dependent effect of neural agrin on the CPD of human myoblasts. *n* = number of Petri dishes; statistical significance: ** *p* < 0.01; *** *p* < 0.001

We also measured BrdU incorporation. Myoblasts derived from three different old and young donors were grown in the presence or absence of 1 nM neural agrin for 24 h. Agrin exposure increased BrdU incorporation in all the three populations of old myoblasts.
Conversely, BrdU incorporation remained unaltered in young myoblasts (Fig. 1B). Thus, the results indicated an age-dependent effect of agrin on myoblast proliferation.

Mini-agrin stimulates BrdU incorporation in old myoblasts

The C-terminal part of agrin is necessary as well as sufficient for the binding to MuSK and α-dystroglycan and for its well-known effect on AChR clustering. To examine whether the effect of agrin resided in this portion of the molecule, BrdU incorporation was evaluated in old myoblasts exposed for 24 and 72 h to 1 nM full-length or mini-agrin, consisting of the C-terminal half of the molecule (see Experimental procedures). BrdU incorporation, expressed as percentage in respect to control, was respectively 19.00 ± 5.57% (n = 7 wells) vs 25.20 ± 4.99% (n = 8) at 24 h and 69.80 ± 9.66% (n = 8) vs 71.20 ± 3.12% (n = 8) at 72 h (Fig. 2). Therefore, mini-agrin mimicked the full-length agrin effect at both the time points considered.

Fig. 2. Mini-agrin mimics the effects of full-length neural agrin in old human myoblasts. (A) Cultured myoblasts from an old donor aged 77 years where exposed to 24 or 72 h treatment with 1 nM mini-agrin. The miniaturized form of neural agrin increased the BrdU incorporation as well as the full-length molecule. Results are mean ± SEM. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001 vs Control.
Results

MuSK is expressed in old and young human myoblasts

MuSK is an essential part of the agrin receptor complex in myotubes\textsuperscript{117,119}. To determine whether MuSK is expressed in human myoblasts, whole-cell lysates were blotted for MuSK, using human and C2C12 myotubes as positive controls\textsuperscript{120}. Western blotting revealed bands of appropriate molecular masses indicating the presence of MuSK receptor in human myoblasts as well as in human and C2C12 myotubes (Fig. 3). Antibody against N-terminal of human MuSK detected two immunoreactive bands between 76 and 100 kDa, in accordance to previous reports\textsuperscript{139}. The bands were appreciable in myoblasts derived from old as well as young donors.

![Image](image.png)

Fig. 3. MuSK and Abl are expressed in old human myoblasts derived from old donors
Representative Western Blot for MuSK and total Abl in human myoblasts and myotubes and C2C12 myotubes. Bands marked with Mb and Mt were from myoblasts and myotubes respectively. Human cells were from a 65 year-old donor. GAPDH was used as a loading control.

Inhibition of Abl downstream of MuSK prevents the effect of neural agrin in old myoblasts

Abl is a well-known effector of MuSK and contributes to agrin-induced AChR clustering in differentiating myotubes and adults skeletal muscle fibres\textsuperscript{140}. More recently, it has been also reported the role of Abl kinase as regulator of muscle cell proliferation\textsuperscript{141}. Therefore, we explored whether agrin required Abl activity to stimulate old myoblast proliferation.

We first assessed the presence of Abl kinase in old myoblasts. Western blotting detected Abl kinase band in old myoblasts, as well as in human and C2C12 myotubes (positive control; Fig. 3).

In a second set of experiments, myoblasts derived from old donors were exposed to a
specific Abl kinase inhibitor, Imatinib (STI571, Gleevec®). A set of control experiments was carried out to check the effect of imatinib applied alone. A 48-h incubation with 1 μM imatinib slightly decreased the BrdU incorporation (95.60 ± 9.23%; n = 3 wells), however the effect was not statistically significant. At 10 μM imatinib, the percentage decreased to 70.73 ± 6.00% (n = 6), with a reduction of ~30% with respect to untreated cells (Fig. 4). Apparently, no toxic effect observed in imatinib-treated cells at the two concentrations (data not shown).

In HM66 treated with 1 nM neural agrin (48 h), the Abl inhibitor prevented the agrin-effect at both concentrations, indicating that Abl activity mediated the effect of agrin on old myoblast proliferation. When co-administered at 1 μM and 10 μM, the percentages of BrdU incorporation were significantly lower than in cells exposed to agrin alone: respectively 107.40 ± 20.44% (n = 3) and 63.43 ± 6.02% (n = 3; Fig. 4).

Fig. 4. Neural agrin depends on Abl to promote old myoblast proliferation. 1 and 10 μM imatinib was used to block the activity of Abl kinase. When applied alone, imatinib significantly decreased the BrdU incorporation at 10 μM. When 1 and 10 μM imatinib were co-administered with neural agrin, the BrdU incorporation was significantly diminished in respect to agrin-treated cells. Results are mean ± SEM. Statistical significance: *p < 0.05 vs Control. #p < 0.05 vs agrin-treated cells.

Agrin depends on ERK1/2 to stimulate myoblasts proliferation

We next investigated whether suppression of ERK1/2 was involved in the proliferative effects of agrin. To do this, we used PD185352, a MEK1 kinase inhibitor preventing ERK1/2 activation. We determined BrdU incorporation in old human myoblasts treated with 1 μM PD185352 in the absence or presence of 1 nM agrin. Cultured myoblasts were
Results

exposed to these treatments for 24 or 48 h prior to BrdU assay. As shown in Fig. 5, PD185352 alone reduced basal BrdU incorporation by 60% (respectively for 24 and 48h: 38.59 ± 6.94%, n = 6 wells, p < 0.001 and 43.75 ± 9.95, n = 6, p < 0.001) and completely prevented agrin-stimulated increase in BrdU incorporation in old myoblasts (respectively for 24 and 48h: 39.52 ± 4.51%, n = 6 wells and 33.91 ± 2.41%, n = 6). Taken together, these data suggest that inhibition of ERK1/2 blocks agrin-stimulated proliferation of old myoblasts.

Fig. 5. Neural agrin depends on ERK1/2 to promote myoblast proliferation. Human myoblasts derived from a 66 years old donor were exposed to 1 μM PD185352 in the absence or presence of 1 nM agrin for 24 or 48 hours prior to BrdU assay. Results are means ± SEM. Statistical significance: **p <0.01, *** p <0.001 vs Control; ### p <0.001 vs neural agrin.

**Exposure to neural agrin does not impair the differentiation efficiency of old human myoblasts**

We also checked whether agrin-treated old myoblasts maintain capability to differentiate despite the enhanced proliferation. To this end old myoblasts were treated with 1 nM neural agrin in proliferative phase and were then analysed for their ability to fuse into myotubes and to establish the skeletal-type excitation-contraction (e-c) coupling mechanism, based on the mechanical link between L-type voltage-operated Ca^{2+} channels and ryanodine receptors.\(^{143,144}\)
To induce cell differentiation, myoblasts were cultivated in low serum conditions for 10 days (see Experimental procedures for further details). Cell fusion efficiency was analysed evaluating fusion index and number of nuclei/myotube. The presence of the e-c coupling mechanism was assessed by a conventional protocol of stimulation using Ca$^{2+}$ imaging\textsuperscript{145}, i.e. measuring the number of cells in which a Ca$^{2+}$ transient was elicited by depolarisation (60 mM KCl) in Ca$^{2+}$-free conditions (2 mM EGTA excess).

The fusion index and the number of nuclei/myotube were similar to control cells (Fig. 6A). The fusion index was $66.20 \pm 5.40\%$ ($n = 5$ optical fields) in differentiated cells derived from agrin-treated myoblasts $58.60 \pm 9.60\%$ ($n = 5$) and in control cells. The number of nuclei/myotube was $7.86 \pm 1.10\%$, ($n = 28$ myotubes) vs $6.12 \pm 1.09\%$ ($n = 16$) respectively (Fig. 6A).

The percentage of cells exhibiting a skeletal-type e-c coupling mechanism was higher in myotubes derived from agrin-treated myoblasts than in controls (Fig. 6B). The percentages of coupled cells were $87.50 \pm 12.50\%$ ($n = 4$ optical fields) vs $58.33 \pm 7.45\%$ ($n = 5$). The difference between the latter two values, although not statistically significant ($p = 0.07$), highlights a trend of increase in the number of coupled myotubes derived from agrin-treated myoblasts with respect to untreated cells.
Fig. 6. Neural agrin does not impair cell fusion and functional differentiation of old donor-derived human myoblasts. A. Fusion index (%) and mean number of nuclei/myotube in human myotubes derived from agrin-treated and untreated cell derived from a 66-year-old donor. The treatment with neural agrin in the proliferative phase did not impair the ability of cells to form myotubes. B. Left, a representative Ca²⁺ response to 60 mM KCl in Ca²⁺-free conditions measured in a coupled cell. Right, a histogram showing the number of responsive myotubes (coupled cells).
Discussion

The main finding of this study is that neural agrin, a nerve derived factor, boosts the \textit{in vitro} proliferative performance of human myoblasts from elderly subjects. Our results confirm the emerging hypothesis that local chemical signals play an important role in rendering the satellite cell niche a permissive environment for an efficient regenerative activity. Furthermore, they shed some new light on the nerve-release molecules contributing to the regenerative potential of skeletal muscle precursors.

The proliferative potential of myoblasts isolated from old and young donors have been compared: \textit{i}) in the same cell culture conditions (20\% of FCS) and \textit{ii}) in cell populations with a comparable (higher than 75\%) and stable number of desmin positive cells. This was made to exclude any possible contribution of different content of trophic factors, present in the serum essential for cell expansion, and/or possible change in the proportion of myoblasts vs fibroblasts or other cell contaminants\textsuperscript{146} in the determination of CPDs in the different cell populations. In absence of neural agrin, all the myoblast populations from old subjects exhibited a significantly lower proliferative capacity, according to very recent reports showing that this occurred when human myoblasts are cultivated in conditions more similar to the physiological environment \textit{in vivo}\textsuperscript{71}. Chronic cell exposure to neural agrin induced a significant increase in the CPDs limited to the old-derived myoblasts. The experiments of BrdU incorporation indicated that such effect was due to an enhancement of the proliferative potential, although our experiments cannot exclude a possible concomitant inhibition of cell death. Further experiments investigating this intriguing potential effect of neural agrin would merit specific investigations.

Interestingly, the treatment of old myoblasts with a miniaturized form of the molecule, called mini-agrin, mimicked the effect of the full-length molecule on BrdU incorporation. Our results indicate that the effect of neural agrin on myoblast proliferation resided in the same portion of the molecule responsible for the canonical biological effects of the neural trophic factor on AChR aggregation. In fact, the mini-agrin we used, consists of the C-terminal half of the molecule, containing a splicing insert at site \textit{z} (9 aa), and the laminin G-like domains 1, 2 and 3, accountable for the binding to MuSK\textsuperscript{138}. In the light of this observation, it was subsequently explored the expression of MuSK. By western blotting old human myoblast samples we were able to detect the receptor. However, the bands
were very weak, while its expression was potentiated following myoblast fusion and
myotube formation. This is in agreement with published data reporting low levels of
expression for cycling rodent myoblasts and up-regulation of the receptor during
differentiation\textsuperscript{120}. Taking into account that mini agrin owns also the binding site for α-
dystroglycan, we cannot exclude their possible contribution to the control of cell
proliferation. Similarly to MuSK, α-dystroglycan role is documented in mature
differentiating skeletal muscle cells\textsuperscript{147}, while less is known about its function in myoblasts.
Western blotting confirmed the presence of Abl in old myoblast samples. The blocker of
Abl imatinib (10 μM) administered alone reduced the BrdU incorporation in aged
myoblast. Analysis of cell survival (data not shown), excluded any toxic effect of the drug.
Instead, such an effect indicated that Abl played a role in the regulation of cell
proliferation in old skeletal myoblasts. As suggested, imatinib could interfere with bFGF
and HGF present in the serum-containing medium necessarily used to maintain myoblasts
in the proliferative phase\textsuperscript{148}. For this reason imatinib effect on cell proliferation was
assessed also at a lower concentration (1 μM), supposed not to inhibit bFGF- and HGF-
dependent cell proliferation\textsuperscript{148}. In this condition, imatinib still prevented neural agrin
effect on BrdU incorporation, indicating that the action of the trophic factor requires Abl
signalling. This evidence underlines once again that, besides its synaptogenic function,
neural agrin is involved in other cell processes at the muscle level and confirms its
pleiotropic role. If on the one hand agrin signalling could regulate the proliferation in
cycling myoblasts, on the other, after the cell cycle arrest, its function could switch to the
control of the neuromuscular junction formation/stabilization and the maturation of
other cell functional properties\textsuperscript{126-128,149}.
As already mentioned, the effect of neural agrin on proliferation was limited to the
myoblasts populations isolated from old donors. However, Abl proteins and the inhibitory
effect of imatinib on cell proliferation were detected also in myoblasts from young
subjects (data not shown) indicating that the Abl signalling contributes to cell cycling also
in the young cells. One possible explanation for the exclusive effect of neural agrin in old
myoblasts could be that the proliferative rate of young myoblasts are intrinsically high
and cannot be significantly enhanced by the neurotrophic factor, while its effect could
become appreciable in cells where the proliferative potential is reduced. In line with this
interpretation, we observed that the enhancing effect of neural agrin on CPDs varied
from 15 to 45% in old myoblasts and the lower was myoblast proliferative capacity in the absence of the trophic factor, the higher was agrin enhancing effect (see Table 1).

Accumulating evidence indicates that mitogenic signals often depend on the activation of the ERK pathway\textsuperscript{150}. The results of ERK inhibition through the PD185352 compound indicate that this MAP kinase could contribute to agrin-induced modulation of cell proliferation. The extent of inhibition of cell proliferation in the presence of PD185352 was considerably high. This fact suggests a possible interference with other signalling pathways controlling cell cycling. Also for this reason, these results have to be considered only preliminary and new functional data on agrin-dependent ERK activation are needed.

The very last observation was that aged myoblasts chronically exposed to neural agrin in the proliferative phase maintained their myogenic potential, exhibiting cell fusion efficiency and developing an e-c coupling apparatus as in control conditions. Thus, a possible hypothetical use of agrin \textit{in vitro} or \textit{in vivo} to improve the proliferative capacity of resident muscle progenitors would not compromise their successful functional differentiation.

Distribution and effect of endogenous neural agrin in \textit{in vivo} skeletal muscle tissue remains to be ascertained. Even though still not proved at the muscle level, the presence of agrin has been detected at perisynaptic and extrasynaptic positions in central synapses\textsuperscript{151-153}, suggesting a potential range of action outside the synaptic cleft, where neural agrin is released. This together with the evidence that, in rodents and humans satellite cells are localized also in the perisynaptic regions and, sometimes, more abundant in that area\textsuperscript{60,154}, opens the door to the intriguing hypothesis that neural agrin could reach at least the satellite cells in the vicinity of the neuromuscular junction.

It is also interesting to mention that the content of neural agrin \textit{in vivo} depends on age. In general, agrin concentration depends on the quantity released from the nerve ending in an activity-dependent manner and agrin cleavage by the neuronal serine protease neurotrypsin\textsuperscript{155}. The reduced physical activity of elderly and massive muscle denervation observed especially in humans\textsuperscript{67,156,157}, are only two of the possible reasons of a reduced content of neural agrin in muscle. More recently, an increased activity of neurotrypsin has been proposed in a subset of human sarcopenic patients\textsuperscript{133} and the increased serum level of one of the fragments generated by neurotrypsin has been proposed as biomarker for sarcopenia\textsuperscript{158}. 
In conclusion, our results suggest that neural agrin is not only an organizer for the appropriate assembly of elements at the level of the junctional area, but also a controller of a wider range of processes related to the differentiation of the entire cell. Cell strategies potentiating the expression of agrin in vivo have been already proposed to rescue symptoms in congenital muscular dystrophy. We can speculate that the enhancement in the content of neural agrin in sarcopenic muscle could contribute to render the satellite cell niche more permissive for the myogenic precursors, improving the regenerative potential of the aged muscle.
During the accomplishment of this thesis, the following papers and abstracts have been published and presented.

**FULL-TEXT PAPERS**


**ABSTRACTS**


8 Ren E, Luin E, Parato G, Lorenzon P, Sciancalepore M, Pavan B, Bernareggi A. Adenosine receptors modulate the autocrine nAChR-driven \([\text{Ca}^{2+}]\text{, spiking activity of in vitro contracting myotubes.} \) 64th Congress of the Italian Physiological Society (SIF), Ancona Portonovo (Italy), 2013.
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Pirkmajer, S., Filipovic, D., Mars, T., Mis, K. & Grubic, Z. HIF-1alpha response to hypoxia is functionally separated from the glucocorticoid stress response in the in


