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# "EXPLOITING VASOPRESSIN SIGNALLING IN MUSCULAR ATROPHY AND DYSTROPHIES"

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*All the wrong (path)ways we followed  light the right one*

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## 1.The thesis explained

This study aims to evaluate the effects of the stimulation of the AVP-dependent pathways on muscular atrophy.
Arg-vasopressin (AVP) has been demonstrated to have a potent effect as myogenic promoting factor both *in vitro* and *in vivo*. In skeletal muscle AVP signaling is mediated by the V1a receptor, whose stimulation results in the activation of PLC and PLD, increased cytosolic calcium concentration and stimulation of CaMK ($Ca^{2+}$/calmodulin-dependent protein kinase) and calcineurin pathways, leading to the formation of multifactor complexes on the promoter of muscle specific genes, thus activating their transcription.
In this study we induced muscular atrophy by local over-expression of TNF and we evaluated the effects of the stimulation of AVP signaling pathways in this condition by over-expressing V1a receptor. TNF is a pro-inflammatory cytokine and it is known to inhibit myogenic differentiation both *in vitro* and *in vivo*, and we have previously demonstrated that the negative effects of TNF *in vitro* is counteracted by AVP treatment. Moreover it has been demonstrated that TNF plays a key role in the activation of the inflammatory pathways mediated by NF-kB and in the stimulation of protein catabolism. For these reasons, we evaluated, in TNF-expressing muscle, the effects of V1aR over-expression in the regeneration process, in the inflammatory status and in protein degradation. Morphological and morphometric analysis demonstrated that the local over-expression of V1aR in the atrophic muscle enhances the cross-sectional area of the regenerating fibers. Since we noted the presence of infiltrating mono-nucleated cells in muscles over-expressing TNF, we investigated the nature of the infiltrate: it is positive for esterase activity, demonstrating the presence of macrophages. Esterase activity is reduced in muscles over-expressing both TNF and V1aR. We further demonstrated that

the up-regulation of NF-kB expression by TNF is reduced by the contemporary over-expression of V1aR. We noted a prevalence of chemokines and cytokines specific of the pro-inflammatory phenotype of macrophages (M1) in the presence of TNF, but an increased levels of antigens and anti-inflammatory cytokines specific for M2 macrophage phenotype in muscle over-expressing both TNF and V1aR. In regards to the regenerative process, the expression levels of early regeneration markers, such as Pax7 and desmin, are up-regulated by TNF, whereas late differentiation markers, such as myogenin and MHC, are down-regulated. The simultaneous over-expression of V1aR up-regulates the expression of both early and late differentiation markers. These data demonstrate that the stimulation of AVP-dependent pathways enhances skeletal muscle regeneration. We also analyzed PI3K/Akt pathway because TNF is known to induce protein degradation. V1 over-expression maintains the phosphorylation levels of Akt and FoxO, at least inhibiting atrogin-1 transcription activated by TNF, thus counteracting protein degradation.

According to these results we are extending our studies to the effects of AVP signaling in muscular dystrophies. Very preliminary data suggest a positive role of AVP even in dystrophic muscles in mdx and Scgβ-null mice, though technical issues have yet to be solved.

## 2. Introduction

### 2.1. Skeletal muscle homeostasis

The capacity of a biological system to maintain the balance of its chemical and physical features, in response to the external stimuli, is fundamental for its own function and survival.
Skeletal muscle tissue represents 40% of the entire body mass and is crucial for movement, posture maintenance and equilibrium, breathing, heat production, carbohydrates and amino acid storage (Kharraz et al., 2013). Skeletal muscle is a dynamic tissue, capable to respond to physiological stimuli and to altered homeostatic conditions, such as atrophy, or severe injuries by activating a regeneration program.
Skeletal muscle regeneration is an evolutionary preserved, multi-step process (Kharraz et al., 2013). The starting event is myofiber necrosis, in which the partial or total sarcolemmal disruption, with a consequent increase in cytosolic calcium ($Ca^{2+}$) concentration, induces calpain-mediated proteolysis. Moreover the release in the extracellular environment of soluble molecules, such as cytokines and chemokines, recruits the circulating inflammatory cells, giving rise to a key event of the regeneration (Charge and Rudnicki, 2004). Cell populations that infiltrate the damaged area are neutrophils (from 1 to 6 hours after damage) followed by macrophages (48 hours after damage). Neutrophils release reactive oxygen species (ROS) and proteases, facilitate phagocytosis and recruit the circulating monocytes. Macrophages are responsible not only for debris removal but also for the secretion of active molecules (cytokines, chemokines and growth factors) that directly influence regeneration (Kharraz et al., 2013; Saclier et al., 2013b; Saclier et al., 2013a).
Macrophages are currently divided in two different phenotypes. M1 macrophages are the first to appear in the damaged area. The M1

macrophages sustain inflammation releasing pro-inflammatory chemokines and cytokines, such as CCL2, IL1β and IL6. M2 macrophages are detected later during skeletal muscle regeneration and are characterized by the expression of CD163 receptor and by the release of anti-inflammatory cytokines, such as IL10 and IL4, finally leading to the resolution of inflammation and to tissue repair (Kharraz et al., 2013). Skeletal muscle regeneration is stimulated by inflammation and it primarily relies on the activation of many stem cell population. The resident pool of stem cell is represented by satellite cells, quiescent cells located under the basal lamina, expressing Pax7 and capable of self-renewal. In addition many other cell types contribute to skeletal muscle regeneration, although at a lower rate: $PW1^{+}$ interstitial cells (PICs), mesoangioblasts, fibro adipogenic cells (FAPs) and, to a lesser extent, also bone marrow derived stem cells (Kharraz et al., 2013; Wang and Rudnicki, 2012). Satellite cells activation is dependent on soluble factors released by the damaged fibers such as FGF (Fibroblast Growth Factor), TGF-β (Transforming Growth Factor), HGF (Hepatocyte Growth Factor) and nitric oxide (NO) (Chargé and Rudnicki, 2004). The production of inflammatory cytokines is fundamental to skeletal muscle regeneration. For example, IL-6 induces both the myogenic differentiation program via p38 (Wang and Rudnicki, 2012) and the proliferation of satellite cells via JAK2-STAT3 (Janus kinase/signal transducer and activator of transcription ) (Schiaffino et al., 2013). IL-4, highly produced by M2 macrophages, activates myoblast fusion leading to the formation of mature myotubes (Schiaffino et al., 2013).

Satellite cells activation implies transcription of MyoD and Myf5 and then of myogenin and MRF4, a family of bHLH (basic helix-loop-helix) transcriptional factors of proteins, also called MRFs (Myogenic Regulatory Factors) (Yusuf and Brand-Saberi, 2012). These transcription factors co-operate with several cofactors binding the promoters of muscle specific genes, such as muscle

creatin kinase (MCK) and myosin heavy chain (MHC) (Lluìs et al., 2006). Finally differentiation of the myogenic cells and their fusion to form mature myotubes lead to new myofiber formation and to tissue repair. At the end of this process the regenerating areas are structurally and functionally identical to the healthy tissue (Chargé and Rudnicki, 2004).

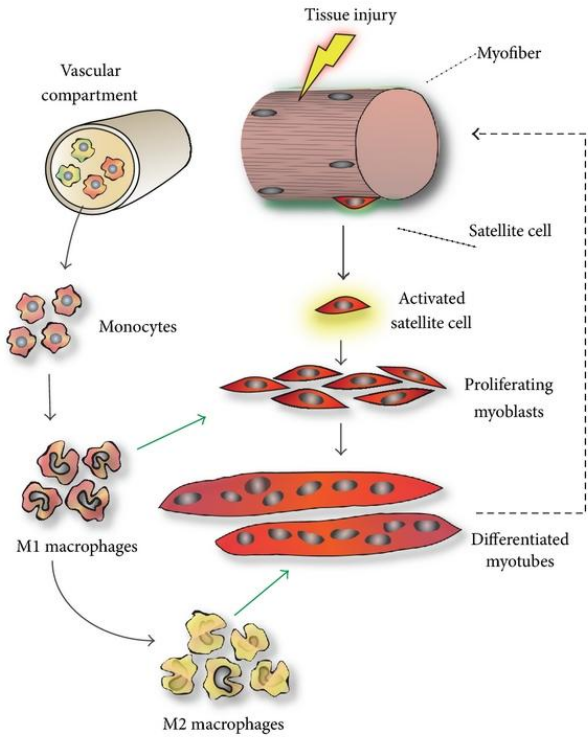


**Figure 1. Inflammation and macrophage polarization in skeletal muscle injury and repair.** (Kharraz *et al*., 2013).

Another important feature during skeletal muscle repair is represented by extracellular matrix (ECM), secreted around every single myofiber by resident fibroblasts. The correct amount and the

spatial distribution of the ECM play a fundamental role as scaffold for new myofiber formation (Kharraz et al., 2013; Mann et al., 2011; Murphy et al., 2012; Serrano et al., 2011). A sustained, persistent inflammation as well as an exhaustion of satellite cells pool, due to extensive injury (more than 20% of the muscle), ageing or altered homeostatic conditions, such as atrophy or dystrophies, lead to fibrotic tissue formation and adipose tissue infiltration, interfering with the correct repair of skeletal muscle (Carosio et al., 2011).

## 2.2. Altered homeostatic conditions: hypertrophy and atrophy

Alteration of the homeostatic conditions leads to both hypertrophy or atrophy of skeletal muscle tissue. Hypertrophy is characterized by increase in protein synthesis, fiber size and number of mitochondria with a consequent enhancement of functionality and contractile force of skeletal muscle (Florini et al., 1991; Florini et al., 1996; Musarò et al., 1999; Musarò et al., 2001; Musarò et al., 2004). This condition can be due to an increased functional requirement or to hormonal stimulation by factors such as IGF-I (Insulin-like Growth Factor I) or utrophin. Muscle specific over-expression of IGF1 in mice enhanced muscle growth and differentiation and maintained muscle mass during ageing (Musarò et al., 2001). It was also demonstrated that IGF1 inhibits protein degradation and muscle atrophy caused by disuse (Schiaffino et al., 2013). In skeletal muscle IGF-I activates the phosphatidite-3-kinase – Akt/ protein kinase B – mammalian target of rapamycin (PI3K-Akt/PKB-mTOR) pathway (Bonaldo and Sandri, 2013; Schiaffino et al., 2013). mTOR forms two complexes: one with raptor (mTORC1) and another with rictor (mTORC2). mTORC1 complex promotes protein synthesis in skeletal muscle via S6 kinase 1 (S6K1) (Le Bacquer . et al., 2007; Schiaffino et al., 2013). S6K1 is

necessary to achieve myofiber normal size (Glass, 2005). mTOR also controls protein synthesis inhibiting PHAS-1, also known as 4E-BP (4E-binding protein), a negative regulator of protein initiation factor eIF-4E (Glass, 2005). mTOR is also involved in the hypertrophic response due to β2-aderenergic agents and androgens (Schiaffino et al., 2013).

PI3K/Akt controls protein balance also regulating FoxO (Forkhead box O) transcription factors. FoxO phosphorylation by Akt maintains these transcription factors in the cytosol, preventing the transcription of the atrophy-related genes, named atrogenes. The up-regulation of atrogenes, that encode for proteins belonging to the ubiquitin-proteasome and autophagy-lysosome systems, leads to muscle atrophy (Bonaldo and Sandri, 2013; Schiaffino et al., 2013). Skeletal muscle atrophy is defined as a decrease in muscle mass that occurs as a result of disuse, starvation, ageing and diseases such as diabetes or cancer (Sishi and Engelbrecht, 2011). Atrophy involves the shrinkage of myofibers due to protein loss, organelles and cytoplasm. In skeletal muscle, atrophy involves several molecular pathways. The proteolysis via Ub-proteasome system is mainly associated with the increased expression of two E3 ubiquitin ligase enzymes: atrogin-1/MAF box and muscle RING finger 1 (MuRF1) (Schiaffino et al., 2013). Atrogin-1 promotes MyoD degradation and, in myogenic cells, interacts with several proteins, such as myosin, desmin, vimentin, enzymes involved in gluconeogenesis and glycolysis, mitochondrial proteins and transcription factors. MuRF-1 controls the half-life of many structural myo-proteins, such as MHC, actin, MLC 1 and 2 and others (Lokireddy et al., 2012; Schiaffino et al., 2013). Protein degradation via Ub-proteasome required an initial cleavage by activated caspase-3 that correlates with the inactivation of PI3K (Kandarian and Jackman, 2006; Wang and Rudnicki, 2012). In catabolic conditions of skeletal muscle is also involved macroautophagy, another important mechanism of protein degradation (Bonaldo and Sandri, 2013; Schiaffino et al.,

2013). Evidences of FoxO involvement also in macroautophagy are given by the correlation between an increased activity of this transcription factor and an increased expression of LC3, the key protein for the formation of autophagosomal vesicles (Bonaldo and Sandri, 2013; Mammucari et al., 2007; Sandri, 2013). Recently another transcription factor, JunB, was demonstrated to block atrophy and to promote hypertrophy: JunB inhibits atrogin-1 and MuRF1, directly binding FoxO3 thus preventing its translocation into the nucleus (Bonaldo and Sandri, 2013; Raffaello et al., 2010; Schiaffino et al., 2013).

Furthermore FoxO1 controls myostatin expression. Myostatin is a member of TGFβ superfamily and one of the key regulator of skeletal muscle mass. It is also demonstrated that myostatin has an inhibitory effect on the IGF1-Akt-mTOR pathway via Smad2/3 (Amirouche et al., 2009; Bonaldo and Sandri, 2013; Sartori et al., 2009; Trendelenburg et al., 2009). Loss-of-function mutations in myostatin gene are known to induce an hypertrophic phenotype in mice, sheep, cattle and humans (Bonaldo and Sandri, 2013). However the role played by myostatin in inducing muscle atrophy is not obvious. The prolonged expression of myostatin leads to muscle atrophy due to up-regulation of ubiquitin ligases (Allen and Unterman, 2007; Lee, 2004; Schiaffino et al., 2013; Taylor et al., 2001) in a FoxO-dependent manner (Bonaldo and Sandri, 2013).

FoxO activity is modulated by the interaction with many co-factors, such as PGC-1α (Peroxisome Proliferator - Activated Receptor Gamma Coactivator 1 alpha) a regulator of energy metabolism in response to external physiological stimuli, such as cold or endurance exercise (Liang and Ward, 2006; Pilegaard et al., 2003). PGC-1α inhibits protein degradation down-regulating FoxO3 activity and NF-κB pathway (Schiaffino et al., 2013).

The Nuclear-factor-kappa B (NF-kB) transcription factor, is one of the most important molecules linked to the loss of skeletal muscle mass in a various physio-pathological conditions. Activation of NF-

kB by pro-inflammatory cytokines, mainly by TNF-α, leads to degradation of specific muscle proteins, induces inflammation and fibrosis, and blocks the regeneration of myofibers (Li and Reid, 2000; Schiaffino et al., 2013). Although the activation of NF-kB itself requires proteolytic processing of IkB family proteins by the ubiquitin-proteasome system (Hayden and Ghosh, 2008; Kumar et al., 2004b), NF-kB can induce expression of FoxO transcription factors by modulating Akt signalling pathway (Mourkioti et al., 2006).

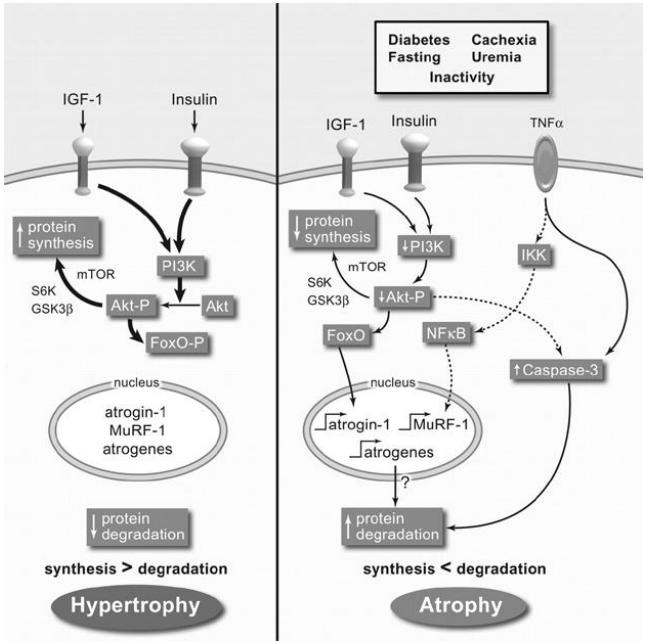


**Figure 2. The balance between protein synthesis and degradation determines muscles hypertrophy or atrophy.** (Lecker et al., 2006).

## 2.3 Neurohypophyseal hormones as non canonical regulators of skeletal muscle homeostasis

Oxytocin (OT) and arginine – vasopressin (AVP), also known as antidiuretic hormone, are two small peptides of 9 amino acid residues synthesized in magnocellular neurons of the paraventricular and supraoptic nuclei of the hypothalamus. They are processed along the axonal projections to the neurohypophysis where they are stored in secretory vesicles and released into the bloodstream in response to a plethora of physiological stimuli (Meyer-Lindenberg et al., 2011).

In women OT is responsible for the ejection of milk during lactation and induces contractions during labor. AVP regulates body's water balance predominantly via its modulatory effects on the expression and function of the water channel aquaporin-2 and of urea transporters in the apical membranes of the epithelial cells of the collecting duct of the kidney nephron. AVP is also a vasoconstrictor that increases peripheral vascular resistance leading to increased arterial blood pressure.

Interestingly, these neuropeptides play unexpected biological roles on many peripheral organs and tissues. Both AVP and OT have a relevant role in cardiac homeostasis. AVP induces cardiomyocytes hypertrophy and is involved in the pathogenesis of heart failure (Hiroyama et al., 2007; Japundzic-Zigon, 2013; Lee et al., 2003). Oxytocin actions include water body balance, general down-regulation of blood pressure and cardiac functions, modulation of parasympathetic system, as well as triggered by the NO pathway modulating vasodilatation, endothelial cell growth and anti-inflammatory response (Gutkowska and Jankowski, 2012).

The role of neurohypophyseal hormones in skeletal muscle homeostasis emerged as a novel subject in the last three decades. Wakelam et al. had shown the presence of functional AVP receptors in the rat myogenic L6 cell (Wakelam et al., 1987). AVP levels

were reported to be very high in developing skeletal muscle, and to decline during gestation reaching a very low level at birth (Smith et al., 1992), suggesting a role for this hormone in muscle development. My laboratory characterized the effects of AVP upon myogenic cells differentiation both in primary cultured and cell lines and analyzed the complex signalling response for such effects (Coletti et al., 2000a; Coletti et al., 2000b; Minotti et al., 1998; Naro et al., 1997; Naro et al., 1999; Naro et al., 2003b; Naro et al., 2003a; Nervi et al., 1995; Scicchitano et al., 2002; Scicchitano et al., 2005; Teti et al., 1993). Breton et al. provided evidence that functional oxytocin receptors are present in human primary myoblasts (Breton et al., 2002; Wakelam et al., 1987). All this observations suggested that neurohypophyseal hormones could play a role during skeletal myogenesis and contribute to maintain skeletal muscle homeostasis.
Their specific action on different tissues and organs is due to the specific expression of several receptors. Both OT and AVP receptors are heptahelical transmembrane proteins activating G proteins. The expression of OTR has been characterized in brain, uterine myometrium, mammary gland, endometrium, decidua, ovary, but also in male reproductive apparatus, thymus, heart and kidney (Gimpl and Fahrenholz, 2001), on the surface of vascular endothelial cells, osteoblasts, cardiomyocytes and human satellite cells (Breton et al., 2002). Three types of receptor are known for AVP: V1a, vascular; V1b, corticotrope, V2, renal. V1aR is located on both vascular smooth muscle cells and cardiomyocytes, have been shown to modulate blood vessel vasoconstriction and myocardial function and it is the only isoform expressed in skeletal muscle. AVP promotes the phosphorylation and desensitization of V1aR. The receptor is internalized in the cytosol, dephosphorylated and rapidly returns to the cellular surface (Birnbaumer, 2000; Koshimizu et al., 2012).

## 2.4 Arginin-Vasopressin effects on skeletal muscle regeneration

The transduction of AVP signal in myogenic cells involves the activation of phospholipases C and D. AVP induces concentration-dependent (0.1 nM - 1 μM) stimulation of phospholipase C (PLC) activity and regulates the intracellular pH with mechanisms involving $Na^+$ and anion transport across the plasma membrane. Inositol 1,4,5-trisphosphate production was maximally stimulated within 2 - 5 sec of treatment with AVP, immediately followed by release of $Ca^{2+}$ from intracellular stores. AVP stimulation of myogenic cells also results in the activation of phospholipase D (PLD) - dependent phosphatidylcholine (PtdCho) breakdown. AVP induces the generation of phosphatidic acid (PA) and the increase of sn-1,2-diacylglycerol (DAG). AVP treatment of myogenic cells also interferes with the cAMP signalling system. It is well known that cAMP-dependent protein kinase (PKA) negatively regulates myogenic differentiation by inhibiting the activity of myogenic Helix-Loop-Helix transcription factors (Li et al., 1992; Winter et al., 1993). In AVP treated L6 cells selective stimulation of specific cAMP-phosphodiesterase isoforms, such as PDE4, in response to PA production by PLD was observed (Nemoz et al., 1997). Consistently, cAMP levels and PKA activity were down-regulated by Vasopressin treatment, allowing the nuclear translocation and the transcriptional activity of Myogenesis Regulatory Factors (MRFs) (Naro et al., 1999; Naro et al., 2003b).

The first evidence of biological effects of neurohypophyseal hormones as modulators of skeletal muscle differentiation came from cell culture experiments. In 1995, Nervi et al. showed that supplementing the culture medium of L6 and L5 myoblasts and of satellite cells with AVP resulted in an important increase of the percentage of fusion and in the formation of hypertrophic myotubes, in the absence of significant effects on cell proliferation. The expression of early and late myogenic differentiation markers was

induced by neurohypophyseal hormones in a structure- and concentration- dependent fashion (Nervi et al., 1995). Using a serum-free culture medium for L6 and L5 myoblasts and for mouse satellite cells (Minotti et al., 1998) demonstrated that AVP effectively induced myogenic differentiation in the absence of other factors. Moreover *in vitro* studies demonstrated that the expression of the V1aR receptor is modulated during myogenic differentiation (Alvisi et al., 2008), which suggests the physiologic involvement of AVP in myogenesis. This hypothesis was also supported by data indicating that both in human and murine embryonic and fetal muscles high levels of immuno-reactive AVP can be detected (Smith et al., 1992). AVP exerts its pro-myogenic effects not only on L6 myoblasts but also on primary satellite cells. In these cells AVP acts in a dose-dependent manner up-regulating the transcriptional levels of Myf5 and myogenin and the expression of muscle-specific genes, such as myosin heavy chain (MHC), muscle creatine kinase (MCK) and acetylcholine receptors (AChR) (De Arcangelis et al., 2005; Scicchitano et al., 2002).

Many transcription factors play a crucial role at the nuclear level during myogenesis. In addition to Myf5, the MEF2 family transcription factors recognize specific DNA elements on the promoter of muscle genes. L6 cells treated with AVP showed an induction of MEF2A and C during myoblast to myotube transition and this induction is concomitant to myogenin expression (Scicchitano et al., 2002). The molecular mechanism of this effect involves the dissociation of transcriptional repressors class-II histone deacetylases (HDACs) from MEF2. Translocation of HDACs II from the nucleus to the cytosol allows the transcriptional activation of MEF2. The AVP-dependent increase of intracellular cytosolic $Ca^{2+}$ concentration activates the CaM-kinase pathway. This is sufficient to induce cytosolic compartmentalization of class-II HDACs, as demonstrated by the reduced nuclear export of the HDACs II and MEF2 activation induced by the treatment of L6

cells with two CaMK inhibitors (Scicchitano et al., 2002). Moreover during AVP-stimulated myogenesis we noted an increase in the acetylation levels of histone H3 associated with the myogenin promoter and the MCK enhancer. However the AVP-dependent full activation of the myogenic program cannot be sustained by CaMK alone (Scicchitano et al., 2005). An additional calcium – dependent pathway involves Calcineurin A (CnA), a serine/threonine phosphatase responsible for the dephosphorylation and the consequent nuclear import of NFAT (nuclear factor activating T-cells) family (Batiuk and Halloran, 1997). Myoblasts treated with cyclosporine A (CsA), a partial inhibitor of CnA, or transiently transfected with the dominant negative construct CnA - KO showed a dramatic down-regulation of myogenic differentiation at both the morphological and the molecular level. Moreover CsA treatment of L6 cells inhibited the expression of MEF2, NFATc1 and GATA2 (a muscle isoform of zinc-finger GATA family), whereas AVP administration promoted accumulation of these three transcription factors into nuclei (Scicchitano et al., 2005). So AVP stimulates the formation of multifactorial complexes including MEF2, NFATc1 and GATA2 in the myoblast nucleus on the promoter or enhancer regions of muscle specific genes via the calcineurin pathway; on the other hand the AVP-dependent activation of CaMK promotes histone acetylation on the same DNA regions, leading to the full expression of the myogenic differentiation program.

Recent work from our laboratory demonstrated that muscle-specific stimulation of AVP signalling induced acceleration of satellite cell activation and earlier stimulation of specific pathways involved in muscle regeneration. The V1aR over-expression in murine injured skeletal muscle was responsible of an anticipation of the regeneration program (Toschi et al., 2011). The rapid recovery of injured skeletal muscle was associated with a rapid resolution of inflammation, earlier activation and fusion of satellite cells, and formation of regenerating fibers. The V1aR over-expressing muscle

already activated satellite cells and markers of muscle differentiation such as Pax7, embryonic MHC, and myogenin, up-regulated CnA signalling during muscle regeneration and presented high expression level of IL-4 (Toschi et al., 2011).
Taken together these data clearly underline the involvement of the AVP-V1aR signalling in skeletal muscle development and homeostasis.

## 2.5. A model of skeletal muscle atrophy

Skeletal muscle atrophy is characterized by loss of proteins, organelles and cytoplasm with a consequent decrease in muscle mass and tissue weakness (Sishi and Engelbrecht, 2011). TNF-α is not only a pro-inflammatory cytokine, but this is also a molecule usually involved in catabolic conditions of skeletal muscle. As demonstrated by Coletti et al. the over-expression of mTNF-α in murine skeletal muscle induced muscle atrophy closely similar to that observed in cancer cachexia (Coletti et al., 2005). It is known TNF-α is able to reduce the number and the size of regenerating fibers and to induce the loss of muscle specific protein in a time- and concentration-dependent manner (Reid and Li, 2001).
At a molecular level TNF-α promotes muscle atrophy stimulating the Ub-proteasomal system, eg. up-regulating the expression of the atrogenes atrogin-1 and MuRF1 (Li et al., 2005). Moreover TNF-α activates JAK/STAT that is sufficient to induce atrophy (Bonetto et al., 2012; Schiaffino et al., 2013). TNF-α signalling also involves NF-κB activation via TRAF1 and 2 (TNF-α receptor associated factors). NF-κB can be a crucial mediator of muscle atrophy, since it induces MyoD degradation and cyclin D1 activation which promotes cell proliferation, not allowing the myogenic differentiation (Coletti et al., 2002; Guttridge et al., 1999). Furthermore TNF-α inhibits myogenic differentiation through the

activation of caspases in a non-apoptotic pathway (Coletti et al., 2002; Moresi et al., 2009). However chronic exposure to TNF-α also induces apoptosis of myoblasts and myofibers, probably due to the activation of caspase-8 (Dirks and Leeuwenburgh, 2006; Stewart et al., 2004).
For all these evidences and in order to study atrophic skeletal muscle, we decided to induce a local, acute atrophy over-expressing TNF-α by gene delivery by electroporation in murine skeletal muscle.

## 2.6. Muscular dystrophies

Lack or impairment of the regenerative capacity of skeletal muscle leads to alterations of tissue physiology. Muscular dystrophies are an heterogeneous group of genetic disease characterized by progressive degeneration of skeletal muscle tissue. Most muscular dystrophies are due to defects in trans-membrane protein complexes which are fundamental for muscle contraction and sarcolemmal integrity. The sarcoglycan complex is a group of single pass transmembrane proteins (α-, β-, γ- and δ- sarcoglycan) that is associated with sarcospan to form a sub-complex within the dystrophin-glycoprotein complex (DGC) in skeletal and cardiac muscles. The DGC includes dystrophin and dystroglycan complexes, which make contact with cytoskeletal actin and laminin in the extracellular matrix respectively (Groh et al., 2009). The expression of the sarcoglycan-sarcospan complex is required for the stabilization of dystroglycan at the sarcolemma and consequently for muscle integrity during contraction. DCG complex plays not only a structural role through the sarcolemma, but it is also a key regulator in signal transduction. Indeed, DCG interacts with nNOS (neuronal nitric oxide synthase) through dystrobrevin and syntrophin (Bonaldo and Sandri, 2013; Sandonà and Betto, 2009;

Yoshida et al., 2000). Dislocation of nNOS from DCG can increase oxidative stress, inflammation and Ub-ligases activity (Lawler, 2011). DCG also interacts with CaMKII (calmodulin-dependent protein kinase II) that phosphorylates several downstream effectors. One of its downstream target is the PI3K/Akt pathway that plays a crucial role regulating skeletal muscle homeostasis (Bhatnagar and Kumar, 2010; Rando, 2001). Many other signalling molecules interact with DCG, such as voltage-gated sodium channels and phosphatidylinositol 4,5-bisphosphate (Bhatnagar and Kumar, 2010; Chockalingam et al., 1999; Gee et al., 1998). Moreover Grg2 (Growth factor receptor-bound protein 2) adaptor protein binds the β-dystroglycan leading to the activation of MAPK (Mitogen-activated protein kinases) signalling cascade. β-dystroglycan also directly associates with ERK1/2 (Bhatnagar and Kumar, 2010; Oak et al., 2001).

Mutations within genes in the DGC complex, such as dystrophin, leads to *Duchenne Muscular Dystrophy* (DMD) or *Becker muscular dystrophy* (BMD), while genetic defects of subunits of the sarcoglycan complex cause *Limb Girdle muscular dystrophies* (LGMD).

DMD is caused by an X-linked mutation in dystrophin gene that leads to the production of non functional protein (Hoffman et al., 1987; Watchko et al., 2002). In humans the onset of the disease is 3 years of age and is characterized by rapid progression which leads to early death due to respiratory or cardiac failure (Cox and Kunkel, 1997; Merrick et al., 2009). The animal model for DMD is the mdx mouse that presents a stop codon mutation in exon 23 of the dystrophin gene (Hoffman and Schwartz, 1991; Watchko et al., 2002). Unlike the human progression of the disease, in mdx mice skeletal muscle undergoes several cycles of degeneration and regeneration of myofibers during 3-5 weeks of life (Pastoret and Sebille, 1995; Watchko et al., 2002).

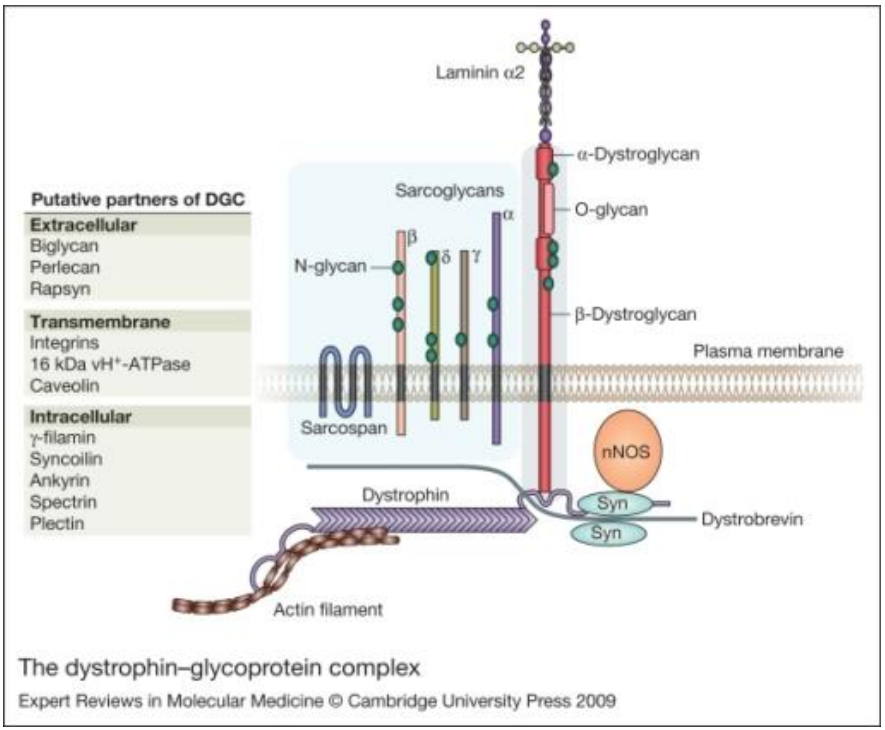


**Figure 3. The dystrophin – glycoprotein complex.** (Sandonà and Betto, 2009)

Inflammation is one of the most important features in muscular dystrophies and NF-κB pathway is a key regulator of inflammation (Aggarwal, 2004; Bhatnagar and Kumar, 2010; Chen et al., 2005). In mdx mice activation of NF-κB and the consequent up-regulation of pro-inflammatory cytokines, such as TNF-α and IL-1β, have been found before the degeneration peak (Bhatnagar and Kumar, 2010; Dogra et al., 2006; Kumar and Boriek, 2003). The inhibition of NF-κB pathway by both single allele deletion and inhibitory peptide reduced inflammatory response and enhanced muscle regeneration in mdx mice (Acharyya et al., 2007). Increased NF-κB activation has been demonstrated also in DMD patients (Acharyya et al., 2007; Bhatnagar and Kumar, 2010; Chen et al., 2005; Monici et al., 2003). MAPK pathways, that include ERK1/2 (extracellular

signal-related kinase), JNKs (c-Jun N-terminal kinases) and p38-MAPK, regulate cell proliferation, differentiation, self-renewal and survival. Activation of ERK1/2 in pre-necrotic diaphragm (Bhatnagar and Kumar, 2010; Kumar et al., 2004a) was demonstrated in mdx mice, as well as the activation of JNK1 and p38-MAPK (Bhatnagar and Kumar, 2010; Kolodziejczyk et al., 2001; Nakamura et al., 2001; Nakamura et al., 2002). Another important molecular pathway that resulted altered in mdx mice is the PI3K/Akt one. Activation of Akt at both pre-necrotic and necrotic stages in mdx has been demonstrated (Dogra et al., 2006). Mdx mice over-expressing Akt showed enhanced muscle regeneration, hypertrophy and decreased sarcolemmal fragility (Peter et al., 2009). Moreover Akt stimulates the expression of utrophin, that partially compensates the lack of dystrophin (Rafael et al., 1998). The Akt-mTOR pathway is also known to modulate autophagy. Recently it was demonstrated that the activation of autophagy by an AMPK (5' AMP-activated protein kinase) antagonist or by stimulating Akt activation ameliorated the dystrophic phenotype in mdx mouse model (De Palma C. et al., 2012). Akt is also implicated in the regulation of intracellular NO synthesis (Dimmeler et al., 1999). It was demonstrated that NO is involved in epigenetic modifications in other cells (Illi et al., 2008). NO deficiency correlating with increased HDAC5 phosphorylation was reported in mdx mice. Phosphorylated HDAC5 is not available to associate with HDAC3, an important complex for satellite cells activation (Colussi et al., 2009). Moreover mdx mice show increased expression of classI HDAC2 and its down-regulation by si-RNA was sufficient to induce morphological amelioration of the dystrophic phenotype (Colussi et al., 2008).

Accordingly to the increased calcium influx (Turner et al., 1991) in dystrophic muscle and to the interaction of DCG complex with CaMK, mdx mice showed an up-regulation in calcineurin-NFAT (Nuclear factor of activated T-cell) pathway. Calcineurin

dephosphorylates the transcriptional factor NFAT, that enters into the nucleus where activates the transcription of muscle specific genes (Olson and Williams, 2000; Schulz and Yutzey, 2004), inducing skeletal muscle differentiation and hypertrophy. Furthermore transgenic mice over-expressing calcineurin showed increased levels of utrophin (Bhatnagar and Kumar, 2010; Chakkalakal et al., 2003).

LGMDs are a group of autosomal recessive disorders that predominantly affect muscles around the scapular and the pelvic girdles. Although the clinical phenotype is very heterogeneous, the disease is characterized by loss of ambulation, difficulties in breathing and premature death (Sandonà and Betto, 2009). Missense mutations in genes encoding β-sarcoglycan give rise to LGMD-2E and results in the absence of the other sarcoglycans and in the reduction of dystrophin and dystroglycan. Mice presenting a substitution of 3-6 exons in Sgc-β gene by neomycin resistance gene represent the animal model for human LGMD-2E. Scgβ-null mice develop muscular dystrophy with hypertrophic appearance and extensive calcification show high levels of serum creatine kinase and myocardial fibrosis (Araishi et al., 1999). Scgβ-null mice show the first degeneration/regeneration phase in skeletal muscle at 2-3 weeks of age and another one at 7-9 weeks, while myocardial lesions become more evident from 20 weeks of age (Araishi et al., 1999).

Little is known about the molecular mechanisms underlying LGMD2E and sarcoglycanopathies in general. In δ-sarcoglycan null mice the Akt activation levels are higher than in age-matched mdx mice (Peter and Crosbie, 2006). In γ-sarcoglycan deficient mice an increased apoptosis is accompanied by the activation of ERK1/2 (Bhatnagar and Kumar, 2010; Griffin et al., 2005).

Beyond the specific differences within each mouse model, these two dystrophic murine models are both characterized by an impairment in the contractile mechanism of skeletal muscle.

Recurrent myofiber damage elicits a constant need for regeneration. In the early phase of the disease the self-renewal potential of skeletal muscle is exhausted, the tissue undergoes a progressive degeneration and is finally replaced by connective and adipose tissue (Meregalli et al., 2013; Sacco et al., 2010).

Many therapeutic approaches have been proposed for skeletal muscle dystrophies, but no one is decisive. Now the most effective treatment is based on corticosteroids administration combined with physical therapy. Corticosteroids have a well known anti-inflammatory effect however other mechanisms of action remain unclear and their use leads to many side secondary effects (Lopez-Hernandez et al., 2009; Sandonà and Betto, 2009). Another pharmacological treatment, successfully applied in mdx mice, is based on amynoglycoside antibiotics that help the stop codon readtrhough (Barton et al., 2002; Sandonà and Betto, 2009). Another gene-based strategy is the exon skipping in order to generate in-frame transcripts, but it is not applicable to sarcoglycanopathies because of the absence of dispensable domains in sarcoglycans (Sandonà and Betto, 2009). Recently the research is focused on cell and gene therapy. The use of adeno-associated viral vectors (AAV) is likely to give results in sarcoglycanopathies employed before the onset of the disease (Allamand et al., 2000; Fougerousse et al., 2007; Sandonà and Betto, 2009). Disadvantages of AAV approach are the low distribution of the vector throughout the tissue and the small size of exogenous DNA that could be inserted (Lopez-Hernandez et al., 2009; Sandonà and Betto, 2009). Cell therapy involves grafting of genetically modified or healthy donor cells. Cells can be satellite cells, bone-marrow derived mesenchimal stem cells, other muscular stem cells, mesoangioblasts and pericytes (Meregalli et al., 2013; Sandonà and Betto, 2009; Wilschut et al., 2012). In sarcoglycanopathies the best result was obtained with satellite cells transplanted in Scgδ null mice by Wallace et al. (Wallace et al., 2008). Injection of mesoangioblasts in

which the expression of α-sarcoglycan was rescued by a lentiviral vector showed a good potential in Scgα null mice (Sampaolesi et al., 2003). Also wild type mesoangioblasts injection in dystrophic dogs resulted in an extensive recovery of dystrophin and led to general amelioration of the pathology (Sampaolesi et al., 2006). Mdx mice treated with mesoangioblasts corrected with a human artificial chromosome containing the human dystrophin gene showed a morphological and functional amelioration of the phenotype for up months after transplantation (Tedesco et al., 2011). Possible disadvantages of cell therapy are the necessity for immunosuppressive treatment and the low diffusion and colonization efficiency (Sandonà and Betto, 2009).

Strategies based on the treatment with IGF-1 or utrophin (an homologous protein of dystrophin) aim to compensate muscle mass wasting promoting hypertrophy and proliferation/activation of satellite cells. The over-expression of IGF-1 in mdx mice led to a recovery of the maximum force (Barton et al., 2002), the viscoelastic properties of the tendon and the number of alive cells (Rizzuto et al., 2013).

The very recent approach based on the administration of histone deacetylase inhibitors (HDACi) to both mdx and Scgα-null mice showed an increased cross-sectional area (CSA) of myofibers, decreases inflammatory infiltrate and fibrotic tissue formation after treatment, unveiling new therapeutic perspective (Consalvi et al., 2011).

## 3. Aims

The goal of this study is to verify whether the activation of the AVP-dependent pathways counteracts muscle wasting in atrophic and dystrophic muscles by enhancing anabolic/regenerative processes and modulating inflammation.
With this purpose, by gene delivery electroporation we locally over-expressed in skeletal muscle of healthy adult mice both the V1a-receptor and TNF-α singularly or in combination.
The strategy of over-expressing the V1aR instead of the Vasopressin directly was employed to prevent the well known systemic effects of AVP. Otherwise we over-expressed only V1aR in dystrophic mice (mdx or Scg-β null mice).

## 4. Results and Discussion

### 4.1. Validation of the experimental model

Previous data of our group demonstrated that muscle specific over-expression of the AVP receptor V1a enhanced skeletal muscle regeneration after CTX induced damage, by stimulating satellite cells activation and increasing the expression of differentiation markers (Toschi et al., 2011). TNF is a well known pro-inflammatory cytokine and it is demonstrated to be sufficient to induce muscle atrophy due to cachexia, as well as inhibition of muscle regeneration (Moresi et al., 2008). Therefore we decided to investigate whether local over-expression of V1aR protects muscle from the effects of high levels of TNF. To this purpose we over-expressed in TA muscles the myosin-light-chain (MLC)-myc-V1a AVP receptor construct alone or in combination with the TNF construct, by means of gene delivery by electroporation. This approach allowed to avoid possible secondary effects of the direct AVP administration. Controls of the experimental model were mock transfected samples (i.d. muscles transfected with pcDNA3) and, when indicated, non-electroporated muscles. In order to assess the efficacy of the transfections we preliminary verified, by Real Time PCR analysis, the V1aR and TNF transcription levels in muscle extracts one week after electroporation (Fig.4A, B). V1aR expression is strongly up-regulated in TA muscles electroporated with MLC-myc-V1a AVP receptor construct, both in the presence and in the absence of TNF, demonstrating that TNF doesn't interfere with the electroporation efficiency or, in general, with the forced expression of V1aR (Fig.4A). TNF transcription levels are high in samples electroporated with the TNF construct alone or in combination with V1aR (Fig.4B).

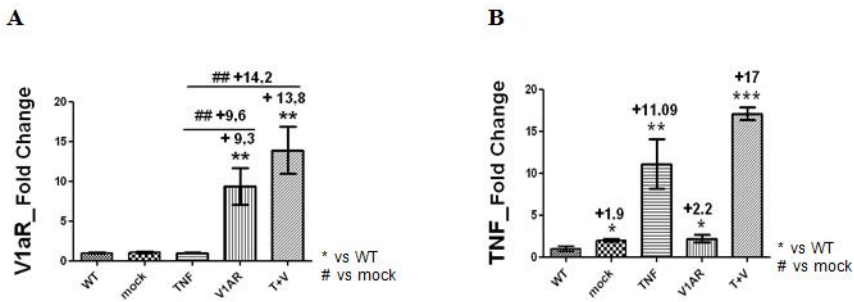


**Figure 4. Electroporation efficiency.** Real Time-PCR analysis for V1aR **(A)** and TNF **(B)** expression in TA muscle, one week after electroporation. *p< 0.05; ** p< 0.01; *** p< 0.001 by Student's t test.

### 4.2. Local V1aR over-expression in skeletal muscle counteracts the negative effects of TNF

Morphological analysis of transfected muscles one week after electroporation shows that local over-expression of TNF (Fig.5A, panel b and f) caused an accumulation of mono-nucleated infiltrating cells. In addition, high levels of TNF induces myofibers damage with a consequent activation of the regenerative process as demonstrated by the number of regenerating fibers in this condition. While mock-transfected and muscle over-expressing V1aR alone didn't show any alteration of the tissue (Fig.5A, panels a and e, c and g, respectively) demonstrating that any damage occurs after electroporation.

As shown in Figure 5B, the number of regenerating muscle fibers was the same in muscles over-expressing TNF alone or TNF with V1aR. Then the co-transfection of V1aR and TNF didn't significantly affects the number of regenerating fibers (Fig.5B), but it affected the fiber size distribution: as shown in Fig.5C, in muscle over-expressing both TNF and V1aR we observed a shift in the size of regenerating myofibers towards a wider cross sectional area (CSA), 200-400 of square micron, compared to the 100-200 square of micron in muscle over-expressing TNF alone.

These data suggest that TNF over-expression induces damage in skeletal muscle, that leads to the activation of the regeneration process. However the V1aR over-expression in presence of high levels TNF leads to more efficient muscle regeneration as suggested by the wider CSA of the myofibers, probably due to an acceleration of the regeneration program.

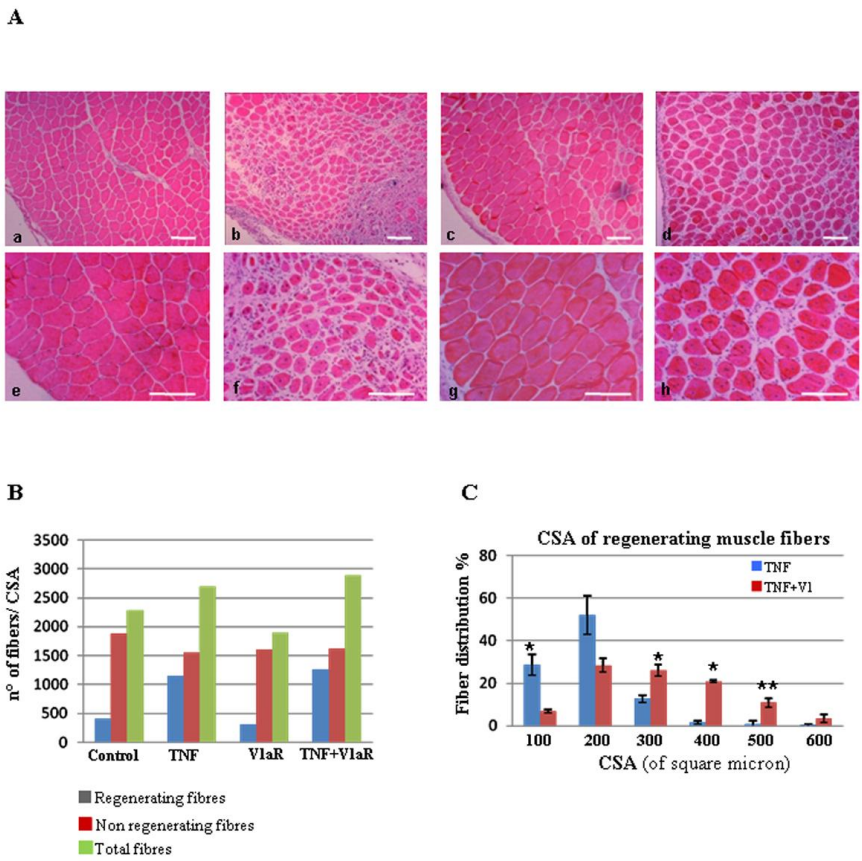


**Figure 5. Muscle over-expressing V1aR in atrophic conditions shows regenerating fibers with a wider area. (A)** H&E stained cross sections of TA of control (panels a and e), TNF- (panels b and f), V1aR- (panels c and g), and TNF+V1aR- (panels d and h) over-expressing muscles performed one week after electroporation. Morphometric analysis of the number of myofiber/CSA divided in regenerating, non regenerating and total for all samples **(B)** and the CSA of the regenerating myofibers of TNF- and TNF+ V1aR- transfected muscles **(C)**. *p< 0.05; ** p< 0.01 by Student's t test.

### 4.3. Local V1aR modulate inflammatory response and fibrogenesis caused by TNF

TNF is a well known pro-inflammatory cytokine capable of activating macrophages and of inducing the production of other pro-inflammatory cytokines, perpetuating the inflammatory response. In order to investigate the nature of the mono-nucleated infiltrating cells observed in the morphological analysis (Fig. 5A) in muscles over-expressing TNF and to verify whether the positive effects of V1aR over-expression is associated with a modulation of inflammation, we examined by aspecific esterase staining the presence of macrophages in our experimental conditions. Fig.6A shows a high number of esterase-positive cells in TNF over-expressing muscles (panel b), whereas the over-expression of V1aR significantly attenuated such increase (panel d). While mock-transfected and V1aR-over expressing muscles were not positive to the esterase staining (panels a and c).

Efficient muscle repair requires the migration and proliferation of fibroblasts in order to produce additional extracellular matrix (ECM) components, which will act as a scaffold for the new myofibers. However, if inflammatory cell infiltration and fibroblast activation persist, an aberrant tissue repair response will produce a non-functional mass of fibrotic tissue. In order to visualize the extent of fibrosis, we performed Masson's trichrome staining in our different experimental conditions. Fig.6B showed absence of connective tissue in mock muscle (panel a) and in muscles over-expressing V1aR (panels c and d), while high levels of TNF were related to the formation of fibrosis in skeletal muscle tissue (panel b).

Taken together these data demonstrate that high levels of TNF induces an inflammatory response and the formation of fibrotic tissue within the muscle, but V1aR over-expression protects

muscles against these effects allowing a more efficient regeneration process.

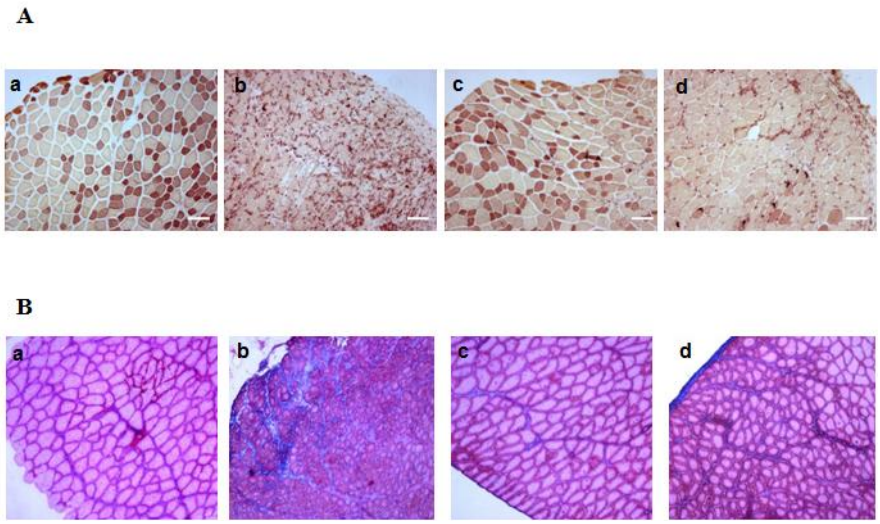


**Figure 6. Muscle over-expressing V1aR counteracts inflammation and fibrosis caused by TNF. (A)** Aspecific esterase stained TA cross-sections of control (panels a), TNF- (panels b), V1aR- (panels c), and TNF+V1aR- (panels d) over-expressing muscles performed one week after electroporation highlighted the presence of macrophages. **B)** Masson's trichrome stained TA cross-sections of control (panels a), TNF- (panels b), V1aR- (panels c), and TNF+V1aR- (panels d) over-expressing muscles performed one week after electroporation shows the presence of fibrotic tissue in muscle.

### 4.4. Modulation of the inflammatory response by V1aR over-expression involves NF-kB pathway

As previous data demonstrated, muscle over-expressing both V1aR and TNF showed less infiltration of mono-nucleated cells and a lower esterase activity if compared with muscle over-expressing TNF alone. Since these observations indicate that the over-expression of V1aR down-regulates the inflammation caused by TNF expression, we deeper investigated the inflammatory response. It is well known that NF-kB pathway is one of the key regulator of the inflammatory system (Smith et al., 1992; Tidball, 2005) and that TNF, along with other pro-inflammatory cytokines, stimulates this pathway (Li et al., 1992). As expected, western blot analysis (Fig.7A-D) revealed that TNF over-expression promoted a down-regulation of the NF-kB inhibitory factor IkBα, with a concomitant up-regulation in the ratio phosphorylated active NF-kB/total NF-kB. Conversely, the phospho-NF-kB/total NF-kB ratio was significantly reduced in muscle over-expressing both TNF and V1aR, with a concomitant increase of IkBα, demonstrating that V1aR over-expression attenuated the effects of TNF on inflammation. Interestingly V1aR alone seemed to down-regulate phospho-NF-kB expression levels with the consequent increase of its inhibitor, suggesting a role of AVP-dependant pathways regulating NF-kB activation in skeletal muscle regardless of TNF effects.

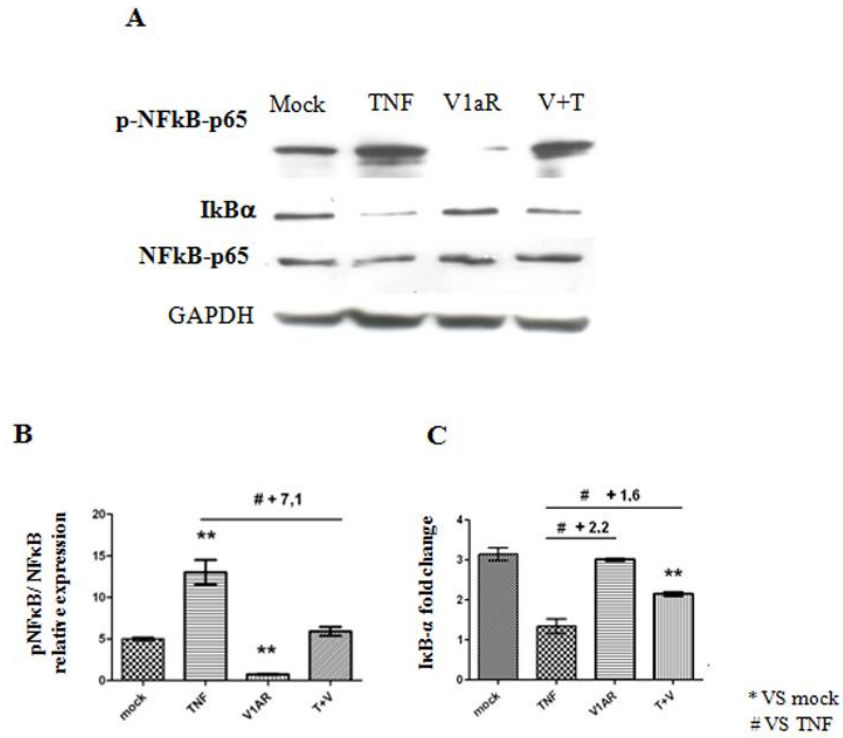


**Figure 7. Modulation of NF-κB pathway in muscle over-expressing V1aR. A)** Molecular analysis of NF-κB pathway one week after electroporation. Over-expression of V1aR contributed to a decrease in the expression levels of the phosphorylated NF-κB and conversely an increase in the expression of IκB. Quantitative analysis in figures **B** and **C**. *p< 0.05; ** p< 0.01 by Student's t test.

### 4.5. Inflammatory cytokine production is modulated by V1aR over-expression

To gain further inside into the mechanism by which V1aR modulates the resolution of inflammation, we investigated by Real Time PCR analysis the expression patterns of specific cytokines and chemokines secreted by macrophages with an M1 phenotype, a pro-inflammatory population capable for perpetuating the inflammatory response (Kharraz et al., 2013). Figure 8 demonstrated that the expression of CCL2 (8A) chemokine, IL1-β (8B) and IL6 (8C) cytokines was strongly up-regulated in the presence of high levels of TNF compared to WT, mock- and V1aR-transfected muscles, while in the presence of both TNF and V1aR their expression was significantly reduced compared to TNF alone (Figures 8A, B and C). Interestingly the reduction in such markers for M1 macrophages, promoted by V1aR over-expression, was associated with an up-regulation of relevant markers of the M2 macrophages, such as the receptor CD163 and IL-10, IL-4 cytokines (Figures 8D, E and F, respectively). These results demonstrated that high levels TNF maintains the pro-inflammatory molecules and macrophage phenotype, perpetuating the inflammation. While V1aR over-expression accelerates the resolution of inflammation caused by TNF expression, favoring the shift of macrophages from M1 to M2 phenotype, stimulating the secretion of anti-inflammatory cytokines and thus promoting regeneration process.

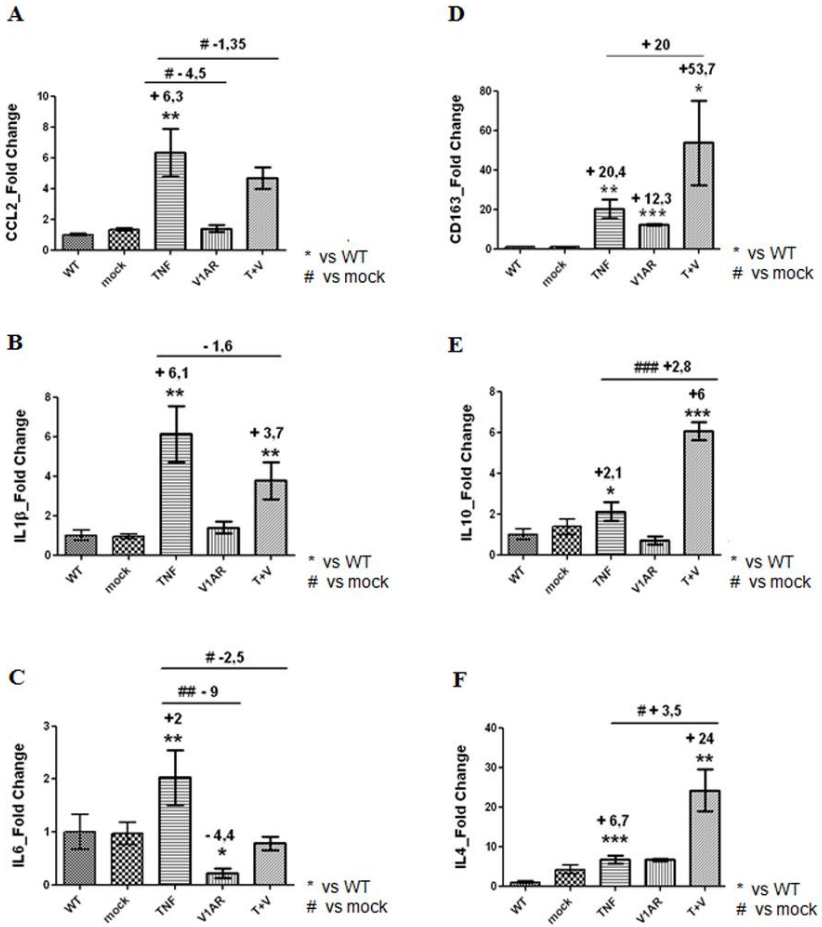


**Figure 8. V1aR over-expression induces the repair phase of inflammation.** Real Time-PCR analysis for CCL2 (A), IL-1β (B) and IL-6 (C) in wild type, mock- , TNF-, V1aR and TNF+V1aR mock transfected muscles one week after electroporation. These chemokines and cytokines are typical of the pro-inflammatory phase and are highly expressed in muscle over-expressing TNF and down-regulated by V1aR over-expression (A). On the contrary real Time-PCR for CD163 (D) receptor and IL-10 (E) and IL-4 cytokines that characterize the resolution phase of inflammation shows an up-regulation in muscles over-expressing TNF+V1aR. *p< 0.05; ** p< 0.01; *** p< 0.001 by Student's t test.

### 4.6. The stimulation of AVP-dependent pathways promotes skeletal muscle differentiation

The ability of AVP signalling to counteract the negative effects of TNF on muscle differentiation was validated in an *in vitro* cell system, the L6 myogenic cells, which are induced to differentiate by AVP treatment (Minotti et al., 1998). Of note, TNF treatment sustained an undifferentiated phenotype, demonstrated by the presence of mono-nucleated myoblasts, by the absence of polynucleated myotubes and by the inhibition of MHC and myogenin expression. Conversely, AVP counteracted the inhibitory effect of TNF and muscle differentiation, up-regulating both myogenin and MHC expression and stimulating muscle growth and maturation (data not shown).

Skeletal muscle regeneration consists of a sequence of phenomena starting from the activation of satellite cells, followed by their differentiation and fusion into myofibers. Therefore we first analyzed one week after TNF and/or V1aR electroporation the expression of Pax7, marker of satellite cells, MyoD and desmin, early markers of myogenic program activation. Western Blot and Real Time PCR analysis demonstrated that TNF over-expression greatly increased the expression levels of Pax7 and desmin, compared to WT and mock-transfected muscles, whereas MyoD expression remained unchanged in all samples. The transfection of V1aR alone did not induce significant expression of these proteins. In contrast, muscles over-expressing V1aR and TNF showed a significant increase in Pax7 and desmin expression (Fig.9 A-C).

We then analyzed the expression of molecular markers characteristic of terminal phase of muscle differentiation, such as myogenin and MHC. High levels of TNF down-regulated the expression of these markers compared to WT and mock-transfected samples, whereas V1aR over-expression enhanced myogenin and MHC expression (Fig.9 D and E). Immunofluorescence analysis for

another marker of late stage of muscle regeneration, embryonic-myosin heavy chain (e-MyHC), showed the expression of this protein in many small regenerating fibers in muscles over-expressing TNF and an accumulation in wider regenerating myofibers in muscles over-expressing both TNF and V1aR (Fig.9 F).

Our hypothesis is that TNF expression stimulates satellite cells activation and so muscle regeneration, but fails to complete the maturation process. In contrast, the over-expression of V1aR counteracts the negative effects of TNF, stimulating muscle growth and maturation.

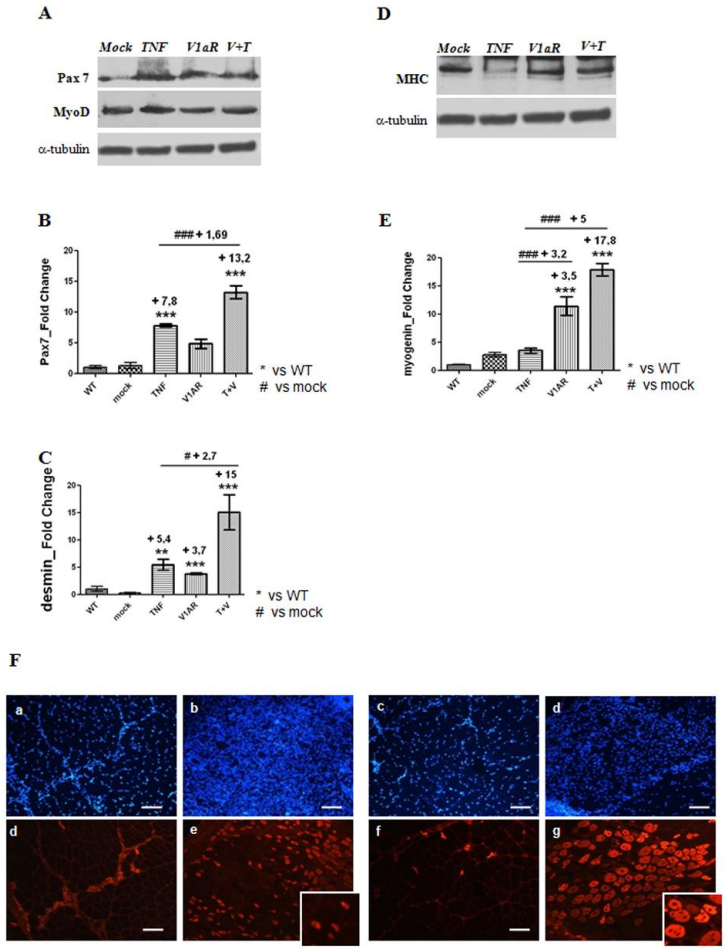


**Figure 9. V1aR over-expression activates skeletal muscle regeneration even in presence of TNF.** Molecular analysis one week after electroporation for the expression levels of Pax7 **(A)**, MyoD **(B)** and desmin **(C)**, markers of early phases of skeletal muscle regeneration in wild type, mock-, TNF-, V1aR- and TNF+V1aR- over-expressing TA muscles. **D and E)** Molecular analysis one week after electroporation for the expression levels of late differentiation markers MHC **(D)** and myogenin **(E)** in wild type, mock-, TNF-, V1aR- and TNF+V1aR- over-expressing TA muscles. **F)** Immunofluorescence analysis for embryonic myosin on TA cross-section of mock- (panel d), TNF- (panel e), V1aR- /panel f) and TNF+V1aR- (panel g) over-expressing TA muscles in red. Nuclei were counterstained with Hoechst in blue (panels a-d), one week after electroporation. *p< 0.05; ** p< 0.01; *** p< 0.001 by Student's t test.

### 4.7. Local V1ar over-expression counteracts the effects of TNF on protein degradation by stimulating PI3K/Akt signalling

We wondered which molecular mechanisms could be responsible for the positive effects of V1aR over-expression on skeletal muscle. Since TNF acts on hypertrophy/atrophy balance in skeletal muscle inducing protein degradation via ubiquitin-dependent proteasome pathway (Li et al., 2005) and since one of the most critical pathway involved in regulation of skeletal muscle mass is the PI3K/AKT one (Bonaldo and Sandri, 2013; Schiaffino et al., 2013), we investigated if V1aR over-expression could modulate this pathway. As shown by Western Blot analysis, in presence of high levels of TNF there was a decrease of phosphorylated form of Akt, with the consequent down-regulation of phospho-FoxO expression levels (Fig.10A.) In this condition p-FoxO enters to the nucleus and activates the transcription of atrogenes, such as atrogin-1, as shown in Real time PCR analysis (Fig.10B). On the contrary V1aR over-expression contributed to maintain at a low level the phosphorylation of Akt and FoxO (Fig.10A), with a consequent down-regulation of atrogi-1 transcription (Fig.10B). In agreement with the literature, TNF over-expression in skeletal muscle stimulated protein degradation, and PI3K/Akt pathway is one of the molecular mechanism involved in. These results demonstrated that V1aR over-expression stimulates PI3K/AKT pathways even in presence of high levels of TNF, leading to phosphorylation of FoxO transcription factors at least resulting in the inhibition of atrogin-1 expression.

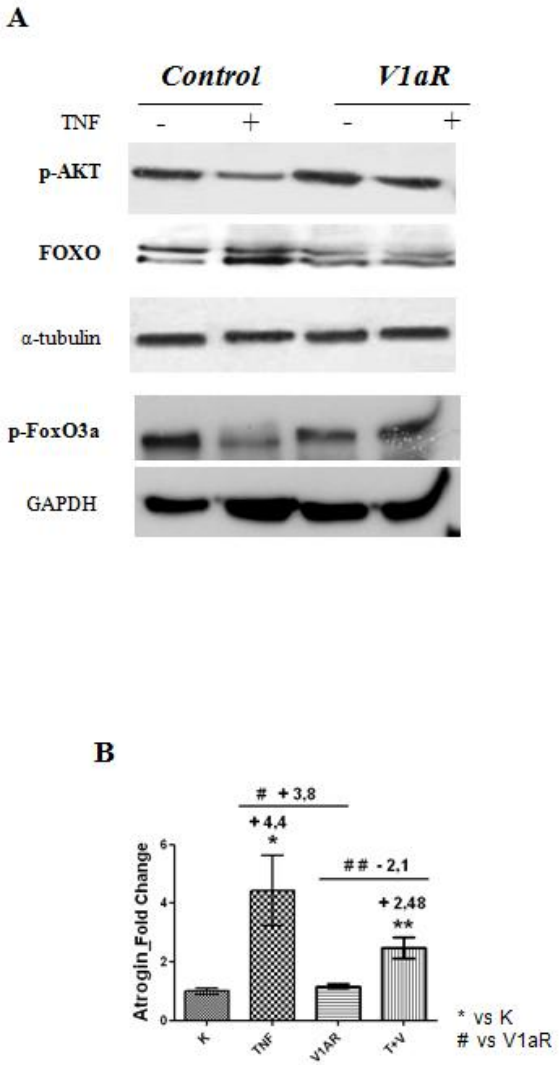


**Figure 10. V1aR over-expression prevents the TNF-dependent modifications of Akt and FoxO. A)** Molecular analysis one week after electroporation of Akt pathway. In muscles over-expressing TNF the expression of phosphorylated Akt and FoxO are down-regulated, whereas V1aR over-expression maintains the phosphorylation levels of the same proteins, preventing FoxO to enter into the nucleus and to transcript atrogin-1, as shown by the Real-Time PCR in **B**, whereas in presence of TNF atrogin-1 transcription is up-regulated. *p< 0.05; ** p< 0.01 by Student's t test.

## 4.8. Validation of gene delivery electroporation in Scgβ null mice

Muscular dystrophies are an heterogeneous group of genetic diseases characterized by muscle wasting and progressive loss of muscle functionality. Since the results obtained in our model of muscular atrophy, we decided to investigate the effects of the stimulation of AVP-dependent pathways by local over-expression of V1aR by gene delivery electroporation in Scgβ null mice, animal model for LGMD (Limb Girdle Muscular Dystrophy). Experiments were performed on 8 weeks old mice because it is demonstrated to be the onset of necrosis/regeneration phase of pathology (Araishi et al., 1999). Controls of the experimental model were wild type (WT), mock transfected (i.d. muscles transfected with pcDNA3) and Scgβ null not electroporated muscles. In order to assess the efficacy of the transfections, we preliminary analyzed the expression of GFP, that was transfected together with V1aR plasmid. As Figure 11. shows, all the transfected samples expressed GFP positive myofibers (Fig.11 panels b, c, e and f), while GFP expression was not detectable in no transfected muscles (Fig.11 panels a and d), demonstrating the efficiency of the technique in this model.

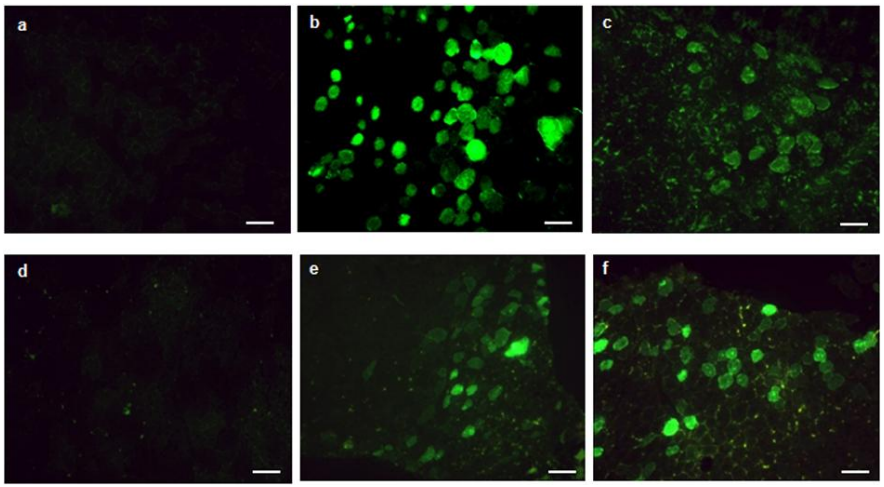


**Figure 11. GFP positive myofibers after gene delivery by electroporation in dystrophic mice.** Analysis for GFP positive cells in fresh cryo cross-section of TA muscle by wild type (upper panels) and Scgβ null mice (lower panels muscles no-electroporated (panels a and d), mock. (panels b and e) and V1aR- (panels c and f) transfected samples shows the efficiency of gene-delivery by electroporation in this animal model one week after electroporation.

### 4.9. Morphological analysis of the effects of Local V1aR over-expression in Scgβ null muscle

Morphological analysis one week after electroporation of mutant (Scg-β null) transfected muscles shows that mock- transfected muscles were subject to extensive damage of myofibers with a consequent infiltration of mono-nucleated cells (Fig.12 panels e - f and e'- f'), if compared to the untreated mutant muscles that showed some necrotic fibers, many regenerating myofibers and less extended infiltration of mono-nucleated cells (Fig.12 panels d and d'). However in Va1R over-expressing muscles (Fig.12 panels f and f') we noted less infiltrate and regenerating muscle fibers with a wider area compared to the mock transfected Scgβ null muscles. All the WT samples had unaltered phenotype (Fig.12 panels a- c and a'- c').

These data suggest that the electroporation caused a damage in Scgβ null muscles, probably due to the intrinsic weakness of a dystrophic muscle, and that the local V1aR over-expression rescues this damage, but its effects on muscular dystrophy are not valuable.

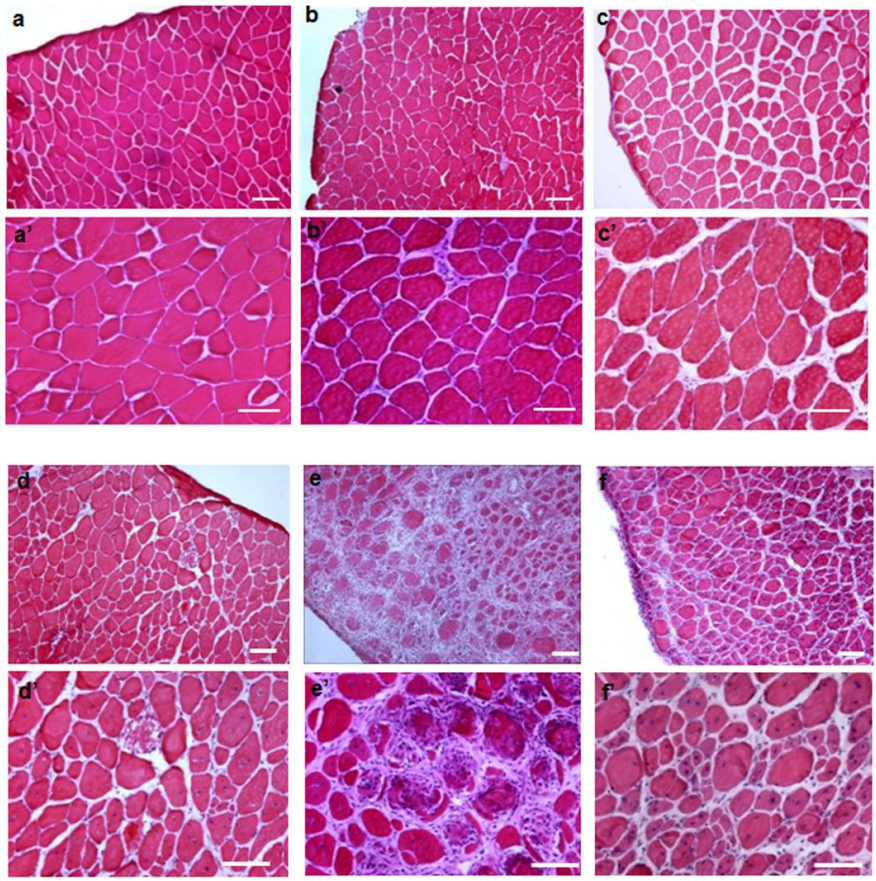


**Figure 12. Morphological analysis of dystrophic muscles over-expressing V1aR.** H&E stained TA cross-sections of wild type (upper panels a-c') and Scgβ null mice (lower panels d-f') of (from left to right) no-electroporated (first column), mock- (second column) and V1aR- (third column) transfected muscles, one week after electroporation.

## 4.10. Effects of local V1aR over-expression in Scgβ null muscle on inflammation and fibrosis

In order to analyze the nature of infiltrating cells observed in mutant muscles, we performed the aspecific esterase staining that highlights the presence of macrophages. As Fig.13 shows, mock transfected mutant muscles appeared highly stained (panels e and e'), V1aR over-expressing mutant muscles showed less esterase stained cells (panels f and f') and only few positive cells can be detected in mutant untreated muscles (panels d and d'). As expected, all the wt samples did not show any macrophage cell (panels a-c and a'-c').
These data confirm the hypothesis of muscle damage due to the electroporation in mutant muscles. Interestingly local V1aR over-expression protected skeletal muscle from the inflammation consequent to this damage, but we noted no modulation of fibrogenesis in these animals. In fact Masson's trichrome staining (Fig.14) shows equal amount of collagen fibers in all mutant muscles not electroporated (panels d and d'), mock- (panels e and e') and V1aR-tranfected (panels f and f'). Wt controls didn't show the formation of fibrotic tissue (panels a - c and a'- c').

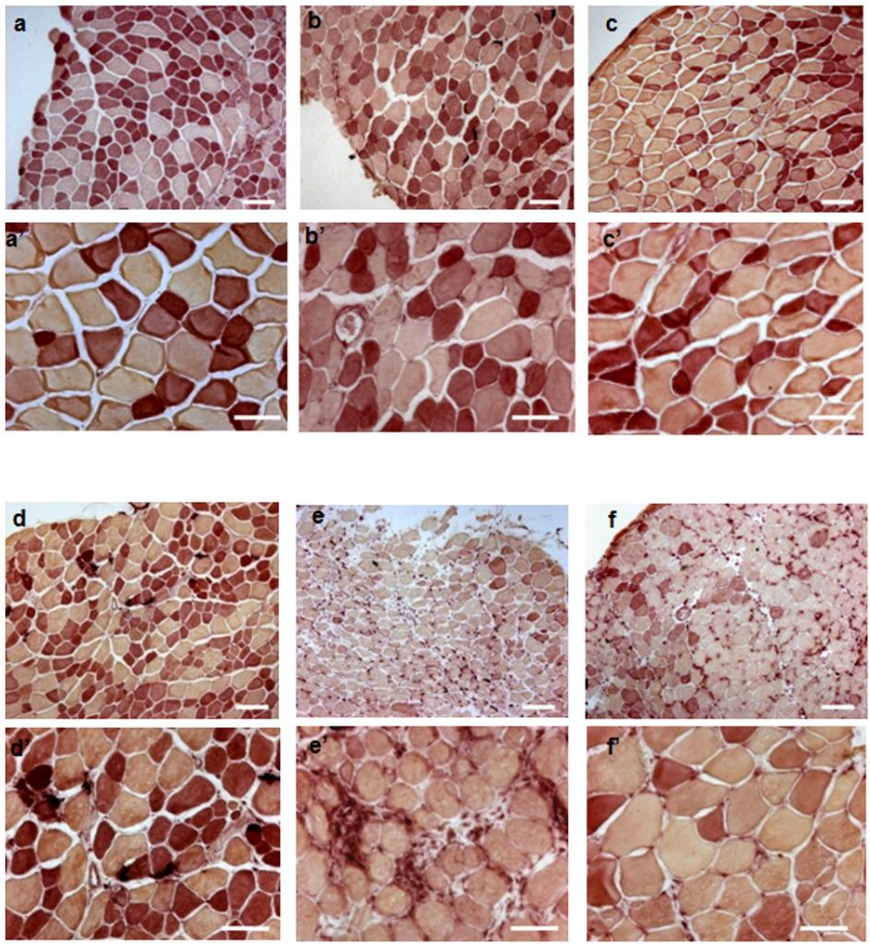


**Figure 13. Analysis of inflammation in dystrophic muscles over-expressing V1aR.** Aspecific esterase staining highlights macrophage activity in TA cross-sections of wild type (upper panels a-c') and Scgβ null mice (lower panels d-f') of (from left to right) no-electroporated (first column), mock- (second column) and V1aR- (third column) transfected muscles, one week after electroporation.

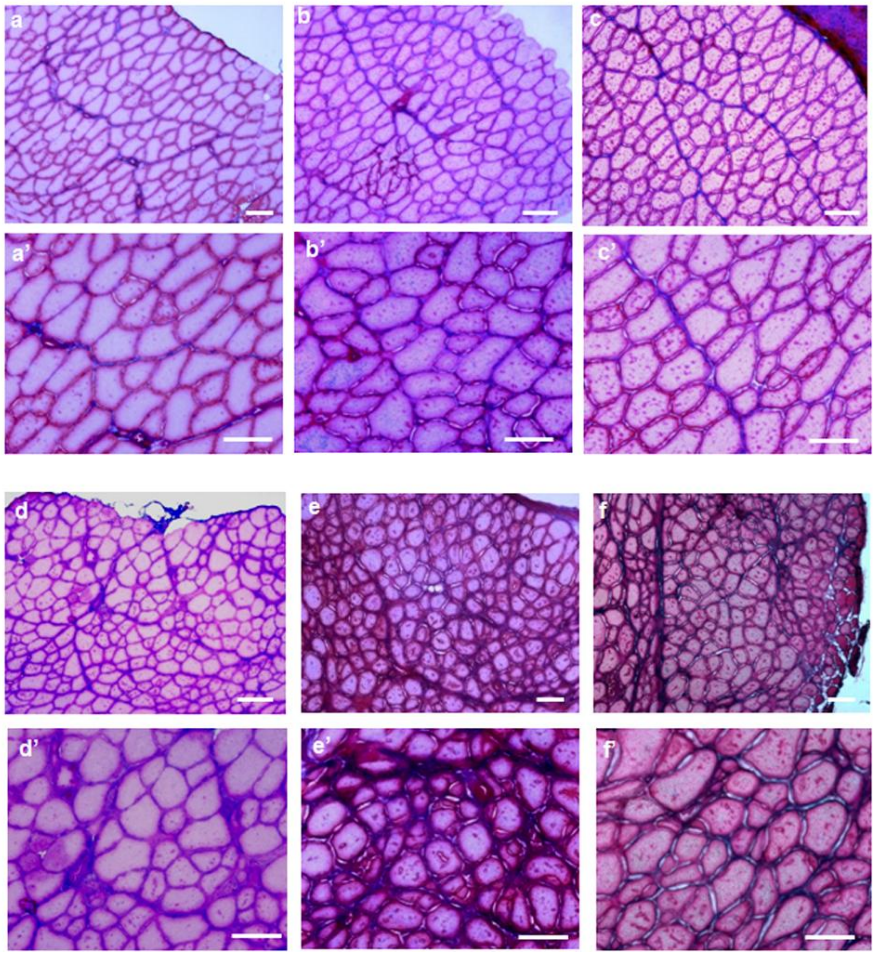


**Figure 14. Fibrotic tissue formation in dystrophic muscles over-expressing V1aR.** Masson's trichrome staining highlighted the formation of collagen fibers in TA cross-sections of wild type (upper panels a-c') and Scgβ null mice (lower panels d-f') of (from left to right) no-electroporated (first column), mock- (second column) and V1aR- (third column) transfected muscles, one week after electroporation.

### 4.11. AVP administration ameliorates skeletal muscle phenotype in mdx mouse model

We decided to investigate the effects of the stimulation of AVP-dependent pathways also in 4 weeks old mdx mice, model of Duchenne muscular dystrophy. Because of the intrinsic weakness of this dystrophic muscles and the early onset of pathology, we cannot perform gene delivery electroporation technique. Thus we analyzed muscles after two weeks of treatment with the direct intramuscular injection of 25µl of AVP 10-7M (0,1 ng/µl) every 48h. As histological analysis shows, we obtained general phenotype amelioration both in the TA muscle (Fig.15A panels b and d), that was directly treated, and in the Gastrocnemius (Ga) (Fig.15B panels b and d) muscle. Contralateral muscles treated with the same protocol but with sterile saline solution are used as control and showed wider infiltration of mono-nucleated cells and smaller regenerating myofibers than the treated muscles (Fig. 15A and B panels a and c).

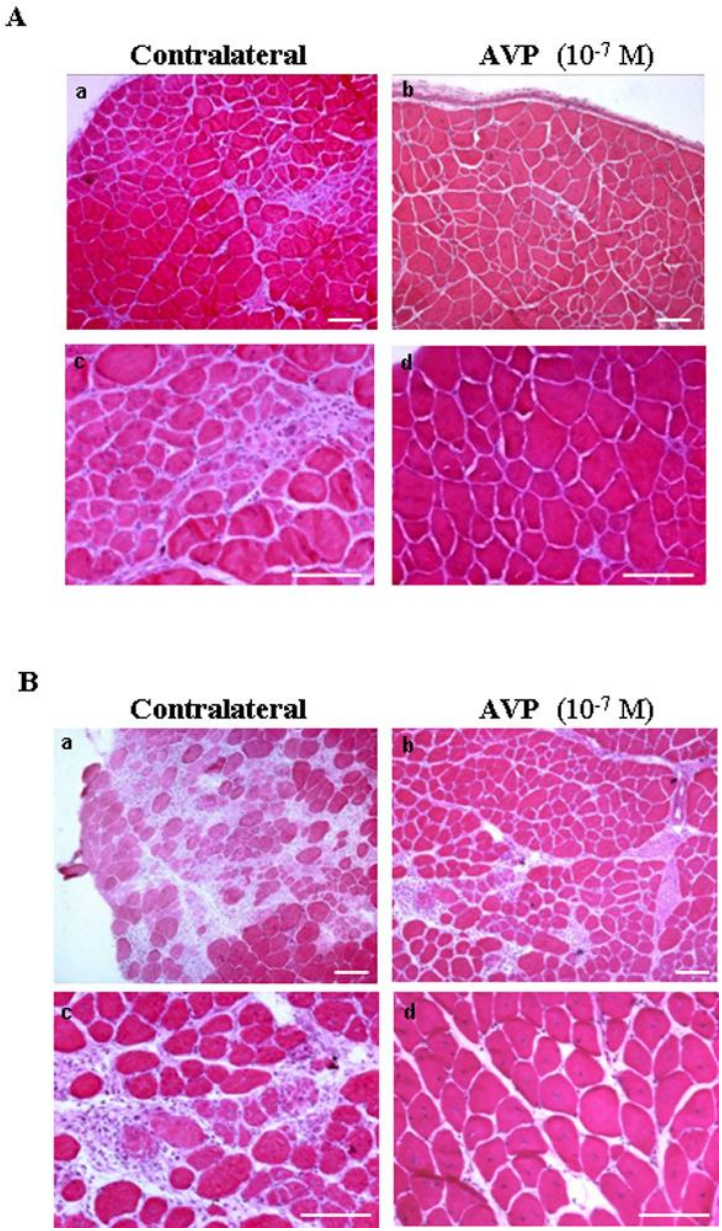


**Figure 15. AVP administration in dystrophic skeletal muscle ameliorates the phenotype. A and B)** H&E stained cross sections of untreated (panels a and c) and AVP $10^{-7}$ M treated (panels b and e) animals of TA **(A)** direct treated muscle and Ga **(B)** muscle after two weeks of treatment.

## 4.12 Discussion

Purpose of this study was to assess if the stimulation of AVP-dependent pathways could have positive effects in an altered homeostatic condition of skeletal muscle: muscular atrophy. Thus we induced atrophic stimuli in TA muscles by local over-expression of TNF and we analyzed the effects of high levels of AVP receptor V1a in this condition. TNF is a pro-inflammatory cytokine and it is known to induce murine myoblast apoptosis (Stewart et al., 2004) and to block human muscle satellite cell differentiation (Foulstone et al., 2004). Moreover, exposure to chronic low levels of circulating TNF inhibits muscle regeneration and induce cachexia (Coletti et al., 2005). AVP is a neurohypophyseal nonapetide and we have demonstrated that it is a potent myogenic promoting factor both *in vitro* and *in vivo*. AVP acts on skeletal muscle by interacting with V1aR. The stimulation of AVP signaling increases cytosolic $Ca^{2+}$ concentrations, that activates both calcineurin and CaMK pathways which lead to the increased expression of myogenic regulatory factors such as Myf5 and myogenin (Nervi et al., 1995; Scicchitano et al., 2002; Scicchitano et al., 2005; Teti et al., 1993). Accordingly local V1aR over-expression results in acceleration of skeletal muscle regeneration after injury (Toschi et al., 2011). Furthermore the calcineurin pathway, which is strongly stimulated by AVP, was shown to be essential for muscle regeneration in normal and dystrophic animals (Stupka et al., 2004). A large body of evidence shows that the AVP system is impaired in several neuromuscular diseases, such as amyotrophic lateral sclerosis and multiple sclerosis (Gonzalez de Aguilar et al., 1999; Michelson et al., 1994). Knock-out mouse for V1aR (V1aR -/-) showed hyperammonaemia due to an increase of protein catabolism, suggesting a role of AVP in the regulation of pathways responsible

for the balance between protein synthesis/degradation (Hiroyama et al., 2007).

Morphological analysis clearly demonstrated that the accumulation of infiltrating cells in muscle over-expressing TNF alone, dramatically decreased when TNF and V1aR are both over-expressed. Moreover, while V1aR didn't significantly modify the number of regenerating fibers compared with samples over-expressing TNF alone, it affected the fiber size distribution. The increased cross-sectional area of the regenerating myofibers in muscle over-expressing V1aR demonstrated a role of AVP in accelerating the regeneration process.

Inflammation is a critical component of muscle physiology and represents an important phase in regeneration (Chargè and Rudnicki, 2004). In particular, the differential expression of M1 or M2 macrophage phenotypes plays a crucial role in the activation of satellite cells (Kharraz et al., 2013). Moreover the inflammatory process is responsible for the formation of new ECM after damage and this assume much importance during the formation of new myofiber in skeletal muscle repair (Hogaboam et al., 1998; Wick et al., 2013). Here we evaluated whether the enhanced regenerative effects observed in V1aR over-expressing muscle correlates with a reduction of inflammation and connective tissue accumulation. Our results clearly demonstrated that the infiltration of monocytes/macrophages and the fibrotic tissue formation observed in muscle over-expressing TNF-α were more rapidly resolved in presence of high levels of V1aR. An attenuation of the inflammatory response has been also evaluated by analyzing the effects of the V1aR over-expression on the activity of NF-κB. NF-κB is one of the most important molecular pathways involved in the activation and maintenance of the inflammation and it is demonstrated to be activated in response to TNF stimuli (Kumar et al., 2004b; Sandri, 2008; Schiaffino et al., 2013). The evidence of an accelerated resolution of inflammation promoted by V1aR over-

expression in our model of muscle atrophy is also demonstrated by its effect on pro- and anti-inflammatory cytokines expression patterns. Local V1aR over-expression selectively down-regulated the pro-inflammatory cytokines, such as IL-1β and IL-6 or CCL2 receptor, whose expression is associated with sustained mononuclear cell influx and is usually up-regulated by TNF stimuli (Kharraz et al., 2013). Furthermore V1aR over-expression correlated with the up-regulation of anti-inflammatory cytokine expression, such as CD163, IL-10 and IL-4 which play a major role in promoting growth and regeneration of skeletal muscle, at least leading to tissue repair (Tidball and Villalta, 2010). Of note the up-regulation of IL-4, that is not only involved in the regulation of inflammatory response, but it is also a molecule able to induce myoblasts fusion during skeletal muscle differentiation (Possidonio et al., 2011). Being directly correlated with CnA activity, IL-4 is synthesized and secreted after the translocation of nuclear factor of activated T-cells (NFAT) into the nucleus and stimulates fusion of myoblasts to myotubes (Charge and Rudnicki, 2003; Horsley et al., 2003; Pavlath and Horsley, 2003). Previous studies of our group demonstrated that IL-4 was also involved in mediating AVP effects on skeletal muscle homeostasis: both *in vitro* in L6 AVP treated cells and *in vivo* after CTX damage the expression of IL-4 resulted up-regulated (Toschi et al., 2011).

Our data demonstrated that local V1aR over-expression counteracts the inflammatory response induced by high levels of TNF and enhances the regeneration process after tissue damage caused by TNF over-expression. We wondering which molecular mechanism could be responsible for the positive effects of V1aR over-expression on skeletal muscle regeneration. We showed that TNF stimulated satellite cells activation and muscle regeneration, as demonstrated by the up-regulation of Pax7 and desmin expression, but, as expected, impinged the maturation process as showing by the low levels of late differentiation markers myogenin and MHC.

In contrast, the over-expression of V1aR stimulated muscle regeneration and maturation, as demonstrating by the expression levels of the same late differentiation markers. We hypothesized that the high levels of TNF compromised the regeneration process and V1aR over-expression counteracted this effect. We speculate that the positive effects on skeletal muscle regeneration after the stimulation of AVP-dependent pathways can be in part due to an enhanced fusion process mediated by IL-4 expression via CnA. This don't exclude an involvement of other pathways or of epigenetic factors, such as a modulation of class II HDACs, whose localization and thus activity is regulated by CaMK, a protein involved in the AVP dependent pathways in skeletal muscle.

TNF is also involved in the activation of catabolic pathways in skeletal muscle. One of the most important mechanisms controlling protein turnover is mediated by Akt-FoxO. The reduction in the activity of the Akt pathway, observed in different models of muscular atrophy, results in decreased levels of phosphorylated FoxO and consequent up-regulation of atrogenes (Sacheck et al., 2007; Sandri et al., 2004) which are responsible for the increased protein degradation through the ubiquitin-proteasome system (Bodine et al., 2001; Gomes et al., 2001; Schiaffino et al., 2013). We demonstrated that TNF over-expression up-regulated the dephosphorylation of FoxO, thus promoting the transcriptional activation of atrogin-1. By contrary, V1aR over-expression stimulates PI3K/AKT pathways leading to phosphorylation of FoxO transcription factors resulting in the inhibition of atrogin-1 expression. So the stimulation of AVP-dependent pathways is also involved in the positive regulation of protein turn-over in skeletal muscle and, in these atrophic conditions, counteracts protein degradation activated by high levels of TNF, maintaining the phosphorylation levels of Akt/FoxO pathway, at least down-regulating atrogin-1 transcription.

In this study we demonstrated that the effects of high levels of TNF on inflammation, fibrogenesis, regeneration and protein degradation in skeletal muscle are strongly counteracted by stimulation of AVP signaling.

Muscular dystrophies are an heterogeneous group of genetic disease characterized by a progressive muscle wasting. Guided by the results observed in our atrophic model, we extended the study to muscular dystrophies, in particular we investigated the effects of the stimulation of AVP-dependent pathways in Scgβ null mice, a model of LGMD2E, and in mdx mice, a model of DMD. Dystrophic muscle show many similarities with atrophic muscle. As in our model of muscular atrophy, also in muscular dystrophies inflammation is an important process, even if here the scenario is dominated by a chronic inflammatory response, mainly due to T cells, that exacerbates muscle wasting (Porter et al., 2002). It was demonstrated that high levels of TNF in muscular dystrophies activate NF-κB pathway, that induces Ub-proteasome system (Charan et al., 2012; Li and Lin, 2008; Mourkioti and Rosenthal, 2008) up-regulating Murf1 and atrogin-1 (Charan et al., 2012; Kandarian and Jackman, 2006; Schiaffino et al., 2013) and down-regulating MyoD expression (Charan et al., 2012; Guttridge et al., 2000). While high levels of TNF in our atrophic model are responsible for the activation of muscle catabolism via Akt-FoxO, in mdx mice PI3K/Akt pathway was demonstrate to be up-regulated, probably for compensating the loss of myofibers, in particular phosphorylated Akt activates mTor that promotes protein synthesis (Peter and Crosbie, 2006).

In our studies on Scgβ null mice we decided to over-express V1aR by gene delivery electroporation in 8 weeks old mice because at this stage muscles are subject to the pick of degeneration (Araishi et al., 1999). V1aR over-expressing mutant muscles showed a general amelioration of muscle conditions if compared to mock-transfected muscle, but not compared to a no-transfected mutant muscle. Thus

the electroporation in this mice induced a damage probably due to the intrinsic weakness of the dystrophic muscles. In our opinion further studies will require less invasive techniques to vehicle the trans-gene in dystrophic muscles. Since in mdx mice gene delivery by electroporation caused too huge damage to muscle tissue at 3 weeks of age, when there is the first cycle of degeneration/regeneration (Pastoret and Sebille, 1995; Watchko et al., 2002), we decided to administrate AVP 10-7M by intramuscular injection. We noted an amelioration of the dystrophic phenotype already at one week of treatment (data not shown); such amelioration is more evident after two weeks. Both the directly treated (TA) and the adjacent muscle (Ga) showed less necrotic fibers, regenerating fibers with a wider CSA and less infiltration of mono-nucleated cells. These data suggest that AVP could exert its positive effects even in the adjacent muscles and not only on the injected muscle. It is not clear whether this effect depends on local or systemic distribution of AVP.

Concluding this study demonstrated that the over-expression of TNF-α in skeletal muscle by gene delivery electroporation is responsible for the stimulation of inflammatory response, demonstrated by the increased number of macrophages; at a molecular level stimulates the activation of NF-κB and the expression of pro-inflammatory molecules typical of the M1 macrophage phenotype. High levels of TNF-α down-regulate the phosphorylation of Akt and FoxO inducing atrogin-1 transcription, leading at least to the activation of muscle catabolism. TNF-induced damage of myofibers activates the regeneration process stimulating satellite cells, however the regeneration doesn't progress beyond the early stages. The stimulation of AVP-dependent pathways by local V1a receptor over-expression significantly counteracts the negative effects of TNF on skeletal muscle. In fact high levels of V1aR in presence of TNF in skeletal muscle inhibited the inflammatory response down-regulating the p-NF-κB and promoting the

expression of cytokines and chemokines typical of M2 macrophagic phenotype and of the resolution of inflammation. V1aR over-expression counteracts TNF-induced effects on skeletal muscle regeneration promoting the progression of differentiation until the late stage of the process. Finally AVP-dependant pathways are also able to regulate muscle metabolism maintaining the phosphorylation levels of the proteins involved in PI3K/Akt/FoxO pathway. Moreover in dystrophic skeletal muscle the stimulation of AVP-dependent pathways exerts positive effects on degenerating muscles, probably enhancing regeneration of myofibers and attenuating inflammatory response.

Our study highlights the positive role of the stimulation of AVP-dependant pathways in atrophic and dystrophic muscle at a morphological and molecular level. Our data can provide a baseline for the future development of strategies to improve muscle regeneration in myopathies.

## 5. Materials and methods

### 5.1 Cell culture

L6 rat myogenic cells were seeded 12.000/cm2 and cultured in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 20 mM Hepes pH 7.4, 50 µg/ml gentamicin solution (Sigma-Aldrich), 2 mM L-glutammine and 10% heat-inactivated FBS (Sigma-Aldrich). Twenty-four hours after plating cultures were shifted to DMEM supplemented with 1% fatty free BSA (Sigma-Aldrich) and treated with 10-7M of synthetic AVP (Sigma-Aldrich) or 5 ng/ml of mTNF-α (Roche, Molecular Biochemicals, Mannheim, Germany) or both of them, for the latter treatment TNF was added 30 minutes after the treatment with AVP.

## 5.2 Animals

C57 transgenic desmin/nls-lacZ mice used in this study bear the 1 kb DNA 5' regulatory sequence of the desmin gene linked to a reporter gene coding for Escherichia coli-β-galactosidase (Lescaudron et al., 1993). The desmin-lacZ transgene labels muscle cells in which the desmin synthesis program has commenced (Lescaudron et al., 1997). Where indicated C57BL/10ScSn-Dmdmdx/J (mdx mice) and B6.129-Scgbtm1Kcam/2J (Scg-beta null mice) mice were also used as models of Duchenne muscular dystrophy and Limb Girdle Muscular Dystrophy (LGMD), respectively. Mice were treated according to the guidelines of the Institutional Animal Care and Use Committee. Animals were anesthetized with an intraperitoneal injection of Avertin A (2,2,2-tribromoethanol and 2-methilbutanol from Sigma Aldrich) before gene delivery by electroporation. After 7 days mice were sacrificed.

## 5.3 Plasmid construction

MLC-Myc-V1aR plasmid used in this work was derived from MLC-Myc and PCD3-V1aR (kindly provided by Prof. S.J. Lolait, Univ. of Bristol, UK) expression vectors, as previously described by Toschi (Toschi et al., 2011). To induce expression of the secreted form of murine TNF-α, we used the construct pBabe-mTNF-α (kindly provided by Dr. Gokhan Hotamisligil, Harvard University, Boston, MA) under control of the SV40 promoter. The SV40 promoter has been demonstrated to be efficient for driving exogenous cDNA expression in skeletal muscle (Lupa-Kimball and Esser, 1998). As transfection control was used SNAP25-GFP plasmid.

### 5.4 Gene delivery by electroporation

The Tibialis Anterior (TA) was injected with the indicated amount of cDNA: 20 µg of MLC-Myc-V1aR, PCDNA3 (Toschi et al., 2011), pBabe-mTNF-α or 10 µg of MLC-Myc-V1aR with 10 µg of pBabe-mTNF-α, in combination with 5 µg of pCMV-SNAP-GFP (kindly provided by Dr. Pozzan, Univ. of Padua, Italy), as a marker of transfection efficiency. The electric pulses were delivered using 3X5 mm Gene Paddles electrodes (BTX, San Diego, CA) placed on either side of the muscle, as described by Donà (Donà et al., 2003). This protocol of gene delivery by electroporation guarantees a stable DNA expression for more than four months.

### 5.5 Histological and histochemical analysis

TA muscles transfected with MLV-Myc-V1aR, PCDNA3 or pBabe-mTNF-α were dissected from 7 weeks old desmin-lacZ mice, embedded in Jung tissue freezing medium (Leica, Wetzlar, Germany) and frozen in liquid nitrogen-cooled isopentane. Cryosections of 7 µm were obtained using a Leica cryostat. Sections were observed under the green activation filter of Axioskop 2 plus system (Zeiss). For histological analysis, sections were stained with hematoxylin and eosin (H&E) using standard methods. Alternatively cryosections were treated for Masson's trichrome staining according to Sigma Aldrich kit protocol.
Esterase staining was adapted from Davis (DAVIS, 1959) as previously reported (Berardi et al., 2008). Cryosections of each muscle were incubated for 5 min in a staining solution containing: 3 mg alpha-naphthyl acetate, 0.375 ml acetone, 6.25 ml 0.2 M sodium phosphate and 0.4 ml of a solution containing equal volumes of 2% pararosaniline (Sigma-Aldrich) and 2% sodium nitrite. Photomicrographs were obtained using an Axioscop2 plus system

equipped with an Axiocam HRc (Zeiss, Oberkochen, Germany) at 1300x1030 pixel resolution and analyzed using 10x NA 0.30 air objective lens or 20x NA 0.50 air objective lens.

### 5.6 Morphometric and statistical analysis

Photomicrographs of the regenerating muscle fibers (identified by morphological criteria, i.e. centrally located nuclei in H&E stained sections) were taken at standard resolution (1.300x1030 pixel) and analyzed using ImageJ, Scion Image software. For the morphometric evaluation of fiber size we analyzed the overall number of regenerating muscle fibers/ TA cross section and we the size of 200 regenerating muscle fibers of the muscles over-expressing pBabe-mTNF-α alone or pBabe-mTNF-α and MLC-Myc-V1aR. All data are expressed as mean ± SD. Statistical analysis was performed by means of Student's t test.

### 5.7 Immunofluorescence analysis

Transverse TA muscle cryosections were fixed in 4% paraformaldheyde for 10 min on ice and washed with PBS. After incubation in 1% BSA (Sigma-Aldrich) for 30 min at RT, cryosections were incubated over night at 4 °C with embryonic myosin (F1.652) from Developmental Hybridoma-bank and then for 1h at RT with the appropriate secondary antibody, Alexa fluor 568-conjucated anti-mouse (Molecular Probes, Eugene, OR, USA) 1:500 in 1% BSA. Nuclei were stained with 0.5 ug/ml Hoechst 33342 (Sigma-Aldrich). The sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined with an Axioskop 2 plus system (Zeiss) or a

Leica Leitz DMRB microscope fitted with a DFC300FX camera for confocal analysis (Leica).

### 5.8 Gene expression analysis

TA muscles were dissected, minced and homogenized. Total RNA was extracted by means of Trizol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. RT-PCR was performed using 1 µg of total RNA that was reverse-transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Invitrogen, Carlsbad, CA).
For Real-time PCR, cDNA was performed from three independent RNA preparation, while quantitative PCR was performed with ABI Prism 7500 PCR instruments (Applied Biosystems) using the TaqMan gene expression assay to amplify samples in triplicate for TNF (Mm00443258-m1) V1aR (assayMm00444092_m1), CCL2 (Mm00441242-m1), IL1-beta (Mm01336189-m1), IL6 (Mm0120733-m1), Cd163 (Mm004744096-m1), IL10 (Mm00439616-m1), IL-4 (Mm00445260-m1) Pax7 (Mm00834082_m1), Desmin (Mm00802455-m1) and Myogenin (Mm00446194-m1), Atrogin-1 (Mm01207878_m1) and GAPDH (Mm00656735-m1) or HPRT (Mm00446968_m1) as internal control.

### 5.9 Immunoblotting analysis

TA muscle were dissected, minced and homogenized with RIPA buffer (20 mM Tris/HCl pH 7.5, 2mM EDTA, 2mM EGTA, 0.25M Sucrose, 5mM DTT, 0,1% Triton X-100, 10mM NaF, 200 µM sodium orthovanadate) and sonicated. Equal amount of proteins (20 µg for cells and 30 µg for muscles), determined by BCA protocol,

were separated by SDS PAGE and transferred electrophoretically to Hybond-C Extra nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Aspecific binding sites were blocked in TBST 5% non-fat milk for 1h. Then the membrane was incubated over night with the monoclonal primary antibodies in 5% BSA/TBST: anti-IL-4 2 μg/ml (R&D system), anti-Pax7 from Developmental Hybridoma-bank undiluted, anti-MyoD (Santa Cruz Biotechnologies) 1:500, anti-MHC (MF20) from Developmental Hybridoma-bank, anti-myogenin F5D from Developmental Hybridoma-bank undiluted, anti-α-tubulin (Sigma-Aldrich) 1:50, anti-GAPDH (Santa Cruz Biotechnologies) 1:10000, or with the polyclonal primary antibodies anti-NFkB (C22B4) and anti-phospho NFkB p65 (Ser563) (93H1), diluted 1:1000 (Cell Signaling), anti-calcineurin Pan A (Chemicon International) 1:500, anti-phospho Akt (Ser473), anti-FoxO3a (75D8), anti-phospho FoxO3a (Ser253) diluted 1:1000. Blots were washed in TBST and then incubated with the appropriated secondary antibody, goat anti-mouse or anti-rabbit HRP-conjugated (Bio-Rad Laboratories, Hercules, CA) in TBST containing 1% non-fat milk. Blots were extensively washed and the antibody binding was detected by Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

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## 7. List of publications

Perniconi, B., Costa, A., Aulino, P., Teodori L., Adamo, S. and Coletti, D. (2011). The promyogenic environment provided by whole organ scale acellular scaffolds from skeletal muscle. Biomaterials 32: 7879 – 82.

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