Exploring the Role of Agrin in Skeletal Muscle Growth

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ABSTRACT

Exploring the Role of Agrin in Skeletal Muscle Growth

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Skeletal muscle growth is important for organism development. It is also important throughout adult life as a result of exercise and to recover from injuries and diseases. Defects in the genetic programming underlying these processes give rise to myopathies. The molecular mechanisms underlying muscle growth are not fully elucidated, but it is known to be triggered by the initial contact between neuron and muscle fiber. This interaction promotes secretion of agrin by the motor neuron to initiate development of neuromuscular junctions. However, recent studies suggest that agrin may play a more prominent role in skeletal muscle growth and development. In this thesis, I characterized this new role for agrin by studying model myoblasts (cultured C2C12 cells) using fluorescence microscopy, immunoblot analysis and qPCR analysis. First, I examined effects of agrin on muscle cell proliferation and differentiation by treating C2C12 cells with recombinant neural agrin for the duration of their differentiation into myotubes. Agrin-treated cells show greater levels of MEF2c and myogenin, markers indicative of differentiation, which correlates with an observed increase in myotube number and area. I then determined that agrin did not use the calcineurin pathway to achieve these phenotypes. Rather, agrin was able to restore growth of calcineurin-inhibited cells, suggesting it targets a different second messenger system. As the Akt/mTOR pathway is critical for muscle growth, I tested whether agrin utilizes this signaling pathway through the use of an mTOR inhibitor, rapamycin. Although the Akt/mTOR pathway was found to be upregulated with agrin treatment, this did not fully account for the effects of agrin on growth and differentiation, suggesting that perhaps another second messenger pathway may be responsible. Although there is much more to explore, this data uncovers a new role for agrin in the development of the early stages of skeletal muscle growth. These new findings allow a better understanding of muscle physiology, and serve as a novel potential target in the treatment of sarcopenia and cachexia.

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LIST OF ABBREVIATIONS

ACh	Acetyl choline
AChR	Acetyl choline receptor
α-DG	Alpha-dystroglycan
AGR	Agrin
BSA	Bovine serum albumin
BTX	Bungarotoxin
Cn	Calcineurin
CNS	Central nervous system
CsA	Cyclosporin A
DAPI	4',6-diamidino-2-phenylindole
DGC	Dystrophin-associated glycoprotein complex
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulphoxide
Dok-7	Downstream of tyrosine kinase-7
eIF3f	Eukaryotic translation initiation factor 3 subunit f
FoxO	Forkhead box O
FBS	Fetal bovine serum
FKBP12	FK506 binding protein 12
GSK3β	Glycogen synthase kinase 3 beta
IGF-1	Insulin growth factor-1
LRP4	LDL-receptor related protein 4
MAFbx	Muscle atrophy F-box
MOPS	3-(N-morpholino) propanesulfonic acid
MRF4	Muscle regulatory factor 4
MSTN	Myostatin
mTOR	Mammalian target of rapamycin
MuRF1	Muscle RING-finger protein-1
MuSK	Muscle specific kinase

Myf5	Myogenic factor 5
MyHC	Myosin heavy chain
MyLC	Myosin light chain
NFAT	Nuclear factor of activated T-cells
NMJ	Neuromuscular junction
p70S6K	p70 S6 kinase
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
RAP	Rapamycin
RCAN1.4	Regulator of calcineurin 1.4
RIPA	Radioimmunoprecipitation assay
RPL13	Ribosomal protein L13
RT	Reverse transcriptase
SDS	Sodium dodecyl sulfate
TBP	TATA-binding protein
TEMED	Tetramethylethylenediamine
TnIs	Troponin I slow

INTRODUCTION

The neuromuscular system is a combination of the nervous and the skeletal muscle systems that work together to perform the primary function of movement and posture. Many diseases are at the core of a dysfunctional neuromuscular system, such as neuropathies, motor neuron diseases, and the spectrum of muscular dystrophies (Dupuis and Loeffler, 2009; Hettwer et al., 2014; Meinen and Ruegg, 2006). The brain controls most movements of the skeletal muscle through the nerves stemming from the spinal cord. NeuroMuscular Junctions (NMJs) are the gaps between the neuron and skeletal muscle fiber. They are not only essential for the body to move (contraction and relaxation) but for growth as well. Skeletal muscles depend on signals (electrical and chemical) from nerves to regulate skeletal muscle growth.

Muscle growth and the neuromuscular junction

The motor unit consists of a somatic efferent neuron that stems from the Central Nervous System (CNS) and a muscle fibre. By releasing neurotrophic factors (agrin, neurotrophin-4, neuroregulin), signaling molecules (calcium, nitric oxide), and a neurotransmitter (AcetylCholine (ACh)) into the NMJ, the motor neuron communicates with the muscle fibre (Angus et al., 2005). Excitation-contraction couplings and growth-related signaling cascades in muscle fibres are the result of such interactions (Fig. 1). Many diseases are associated with dysfunctional NMJs that ultimately lead to atrophy caused by the loss of a growth stimulus. Several muscular dystrophies are caused by a nonfunctional or missing protein that compromises the structural and functional integrity of the NMJ and thus the muscle fibre (Burden et al., 2013; Deschenes, 2004; Dupuis and Loeffler, 2009; Meinen and Ruegg, 2006; Murray et al., 2012; Valdez et al., 2010). The aforementioned neurotrophic factor agrin has been linked to play a major part in many of these diseases (Dupuis and Loeffler, 2009; Hettwer et al., 2014; Meinen and Ruegg, 2006).

Agrin

Agrin is a large (217 kDa) heparan sulfate proteoglycan expressed in liver, kidney, lung, retina, brain, spinal cord, and skeletal muscle (Ferns et al., 1993; Hoch et al., 1993). Agrin has been shown to play a role in regulating growth of many of different tissues: it regulates hypertrophy and differentiation of chrondrocytes to generate connective tissue within the musculoskeletal system (Hausser et al., 2007), and mediates dendritic growth of hippocampal neurons within the central nervous system (Mantych and Ferreira, 2001; McCroskery et al., 2006). However, agrin is best characterized for being essential for the development and stabilization of NMJs (Bezakova and Ruegg, 2003; Eusebio et al., 2003). Godfrey et al. (1984) and Nitkin et al. (1987) were the first to discover agrin as a neurotrophic factor that was sufficient for pre- and post-synaptic assembly of the NMJ. It was later shown that agrin induces aggregation of AcetylCholine Receptors (AChRs (Ferns et al., 1993)) – hence the name agrin - an early trigger for NMJ development. Agrin has been associated with several diseases such as myasthenia gravis (Burden et al., 2013; Liyanage et al., 2002; Takamori, 2012), congenital myasthenia syndrome (Meinen and Ruegg, 2006) and some forms of muscular dystrophy (Eusebio et al., 2003; Gramolini et al., 1998; Michel et al., 2004). Diseases caused by compromised NMJ include Duchenne Muscular Dystrophy (DMD; (Gramolini et al., 1998) and sarcopenia (Hettwer et al., 2014) among many others. Each of these diseases cause muscle atrophy. Although the underlying mechanisms that mediate disease caused by mutated agrin are largely unknown, the similarity in diseases caused by NMJ dysfunction and mutated agrin strongly suggests that agrin is crucial for NMJ function and muscle growth.

Agrin has four splice variants, three of which are located at the C-terminal region and are known as the X, Y, and Z sites. One is located at the N-terminal which generates a short or long protein (Ferns et al., 1993). The longer N-terminal variant binds laminin and is localized in the basal lamina, while the shorter isoform is incapable of binding laminin and found in the nervous system (Bezakova and Ruegg, 2003). The C-terminal end of agrin has been shown to have AChR clustering activity (Ferns et al., 1993). Alternative splicing at the X site can lead to an insertion of 3 or 12 amino acids, neither of which is tissue specific or affect the biological activity of agrin (measured by AChR clustering). Alternative splicing at the Y site can lead to an insertion of 4

amino acids which has tissue specific expression, predominantly in neuronal tissues (Ferns et al., 1993; Hoch et al., 1993). Lastly, alternative splicing at the Z site can lead to an insertion of 8, 11, or 19 amino acids and has been shown to affect the biological activity of agrin and the assembly and stabilization of the NMJ. The splice variant with the 8 amino acid insert in particular has been shown to be essential to form AChR aggregates at the post synaptic muscle surface (Ferns et al., 1993). The effectiveness of agrin to cluster AChR varies depending on the amount of amino acids inserted at the Z site; the order is $8 \geq 19 > 11 > 0$. The nomenclature used to describe a certain isoform is $AGR_{X,Y,Z}$, where the number of amino acids spliced into each site is written. A recombinant C-terminal half of agrin ($AGR_{3,4,8}$) was used for my thesis to explore its role in skeletal muscle growth.

Agrin at the neuromuscular junction

Agrin is responsible for the stabilization and organization of molecules at the NMJ to ensure that there are efficient communications between nerves and muscles. Once it is released by the motor neuron into the synapse, it can bind to either of two receptors: α-DystroGlycan (α-DG) and LDL-receptor Related Protein 4 (LRP4, see Figure 2; (Hoch, 1999). Binding to α-DG promotes aggregation of the Dystrophin-associated Glycoprotein Complex (DGC; (Hoch, 1999). The main function of the DGC is to maintain the NMJ. It also serves as a scaffold for important signaling molecules (Pilgram et al., 2010), as well as for AChRs to promote formation of NMJs (Campanelli et al., 1994). Specifically, the agrin - α-DG interaction promotes rapsyn binding and the downstream assembly of actin cytoskeleton through utrophin and the recruitment of AChRs (Zhou et al., 1999). To help stabilize the interaction between the neuron terminal and myotube, the amino-terminal end of agrin interacts with laminin-211 (Yoshida-Moriguchi et al., 2010). Finally, agrin can also bind the LRP4/Muscle Specific Kinase (MuSK) complex, which also contributes to AChR recruitment (Kim et al., 2008; Zhang et al., 2008).

Although agrin plays an essential role in NMJ formation and stability, new studies suggest that it may have additional roles in skeletal muscle growth. For example, over-expression of the agrin C-terminal domain in a transgenic mouse model of disease was sufficient to overcome the pathology of congenital muscular dystrophy (Meinen and Ruegg, 2006). Agrin

can also improve whole body health in diseased mice, restore muscle integrity, and improve contractile functions of dystrophic muscles. Agrin may also alleviate the muscle pathology in DMD by increasing the expression of utrophin, a structural and functional homolog of dystrophin (Gramolini et al., 1998). Utrophin transcription is upregulated by many transcription factors including Nuclear Factor of Activated T-cells (NFAT) which is downstream of calcineurin (Cn; Dunn et al., 1999). In addition, agrin stimulates the PhosphatidylInositol-4,5-bisphosphate 3-Kinase (PI3K)/Akt signaling pathway through LRP4/MuSK (Schmidt et al., 2012). Like Cn/NFAT signalling (Dunn et al., 1999; Friday et al., 2000), PI3K/Akt signalling is a major contributor to skeletal muscle growth (Pallafacchina et al., 2002).

Signaling pathways that regulate muscle growth

Skeletal muscle growth can occur via two mechanisms: hypertrophy and hyperplasia (Horsley and Pavlath, 2004). Hypertrophy is a growth in volume, where there is increased protein synthesis and the muscle fiber increases in size. Hyperplasia is a growth in the number of muscle fibers with a relative stability in size. For an *in vitro* system, myoblasts, stem cell-like muscle cells, must first differentiate into myotubes, multi-nucleated muscle cells, before their growth can take place. This process is called myogenesis (Horsley and Pavlath, 2004). *In vivo* muscle cells can undergo myogenesis during their embryonic phase or for muscle reparation after incurring an injury. This can occur due to satellite cells which become active for muscle reparation and must differentiate and fuse into the existing muscle fiber. Altogether, muscle cell differentiation and growth are important for muscle fiber development.

Skeletal muscle growth is dependent on the balance between anabolic (hypertrophic) and catabolic (atrophic) processes. Skeletal muscle hypertrophy is mediated in large part by the IGF/Akt/mTOR pathway (Rommel et al., 2001): Insulin Growth Factor-1 (IGF-1) binds to the IGF-1 receptor (IGFR), which activates PI3K, leading to the phosphorylation of Akt (Lai et al., 2004). Phosphorylated Akt then activates the mammalian Target Of Rapamycin (mTOR), which in turn phosphorylates p70 S6 kinase (p70S6K) to upregulate protein synthesis and growth (Lai et al., 2004). To amplify this pathway, Akt also phosphorylates and inhibits Glycogen Synthase Kinase 3 Beta (GSK3β) a negative regulator of hypertrophy (Cross et al., 1995). Inactivation of

GSK3β blocks export of NFAT from the nucleus, potentially mediating crosstalk between the IGF-1/Akt and Cn/NFAT signalling pathways (Rommel et al., 2001). Then, Cn dephosphorylates NFAT, allowing it to enter the nucleus and activate transcription required for hypertrophy, hyperplasia and differentiation underlying skeletal muscle growth (Dunn et al., 1999).

Contrasting hypertrophy, atrophy can occur in skeletal muscles during disuse, cachexia, and denervation. Many transcripts that are upregulated during atrophy are repressed under IGF/Akt-mediated hypertrophy, e.g. Muscle RING Finger 1 (MuRF1) and Muscle Atrophy F-box (MAFbx, also known as Atrogin-1), E3 ubiquitin ligases that induce atrophy by facilitating protein degradation (Bodine et al., 2001; Gomes et al., 2001). Proteins targeted by these E3 ligases include Myosin Heavy Chains (MyHCs), Myosin Light Chains (MyLCs), MyoD, and Eukaryotic translation Initiation Factor 3 subunit F (eIF3f; (Clarke et al., 2007; Csibi et al., 2009; Tintignac et al., 2005). Importantly, Akt phosphorylates and inhibits forkhead box O (FoxO) proteins – key upstream regulators of atrophy – by preventing entry into the nucleus (Li et al., 2007; see Figure 4). Thus, Akt is a central regulator in skeletal muscle growth and a possible downstream target of agrin.

Central hypothesis and approach

Given that agrin is suggested to have additional roles in muscle development, and that these processes require IGF-1/Akt/mTOR or Cn/NFAT signaling, I hypothesize that agrin is critical for muscle growth and differentiation, processes mediated by the IGF-1/Akt/mTOR and Cn/NFAT signaling pathways. To test this hypothesis, I used fluorescence microscopy, immunoblot analysis, and qPCR analysis to study proliferation and differentiation of C2C12 cells. These cells are a murine cell line capable of differentiating into myotubes (equivalent of an immature myofibre *in vivo*) and thus commonly used as an *in vitro* model of muscle growth. To identify the signaling pathways responsible for the observed effects of agrin on muscle growth, I treated these cells with chemical inhibitors that target signaling pathways of interest on different days of differentiation. Since Cn/NFAT pathway is most important at the early stages of myotube development, I treated the cells at Day 0 and termed it the Cn model. Since IGF-1/Akt/mTOR pathway is most important once the myotubes have already formed, I treated the

cells at Day 2 and termed it the mTOR model. Discovering more of the roles of agrin in skeletal muscle tissue can be advantageous to help understand and treat muscle wasting diseases such as sarcopenia and cachexia.

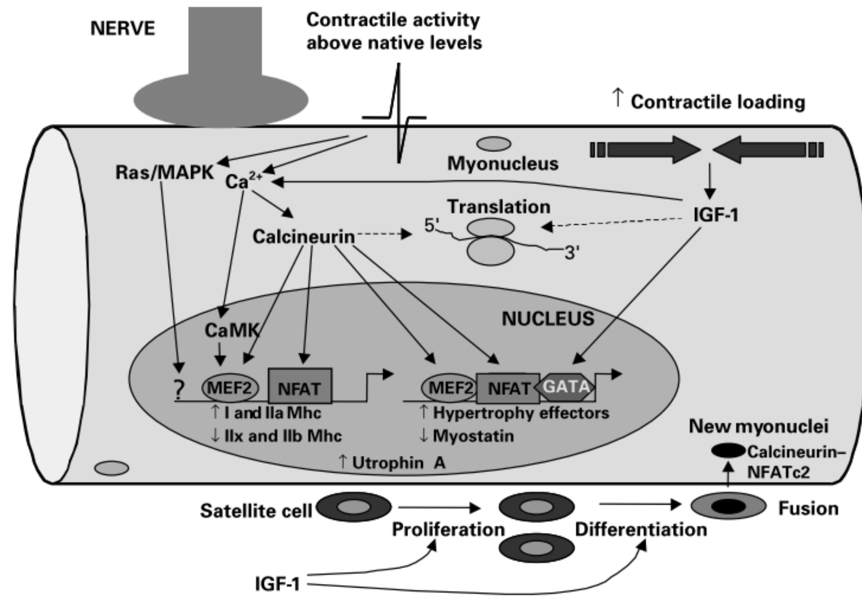


Figure 1. Skeletal muscle growth signaling cascades at the neuromuscular junction
An efferent motor neuron stemming from the CNS comes in contact with a skeletal muscle fibre and can induce a contraction. This leads to several molecular signaling cascades becoming active and leading to growth (from **Michel, Dunn et al. 2004)**.

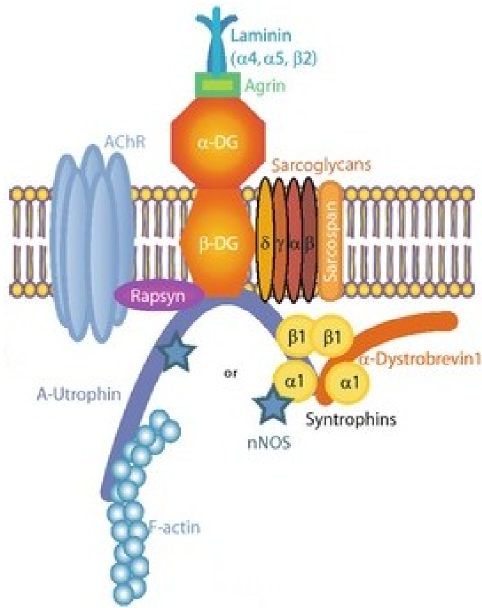


Figure 2. Dystrophin-Associated Glycoprotein Complex at the neuromuscular junction
The DGC is an important protein complex that helps the neuron communicate with the skeletal muscle fibre. It also allows structural stability to the muscle cell membrane. Mutations in the DGC cause a variety of muscular dystrophy disorders (from **Pilgram et al., 2010)**.

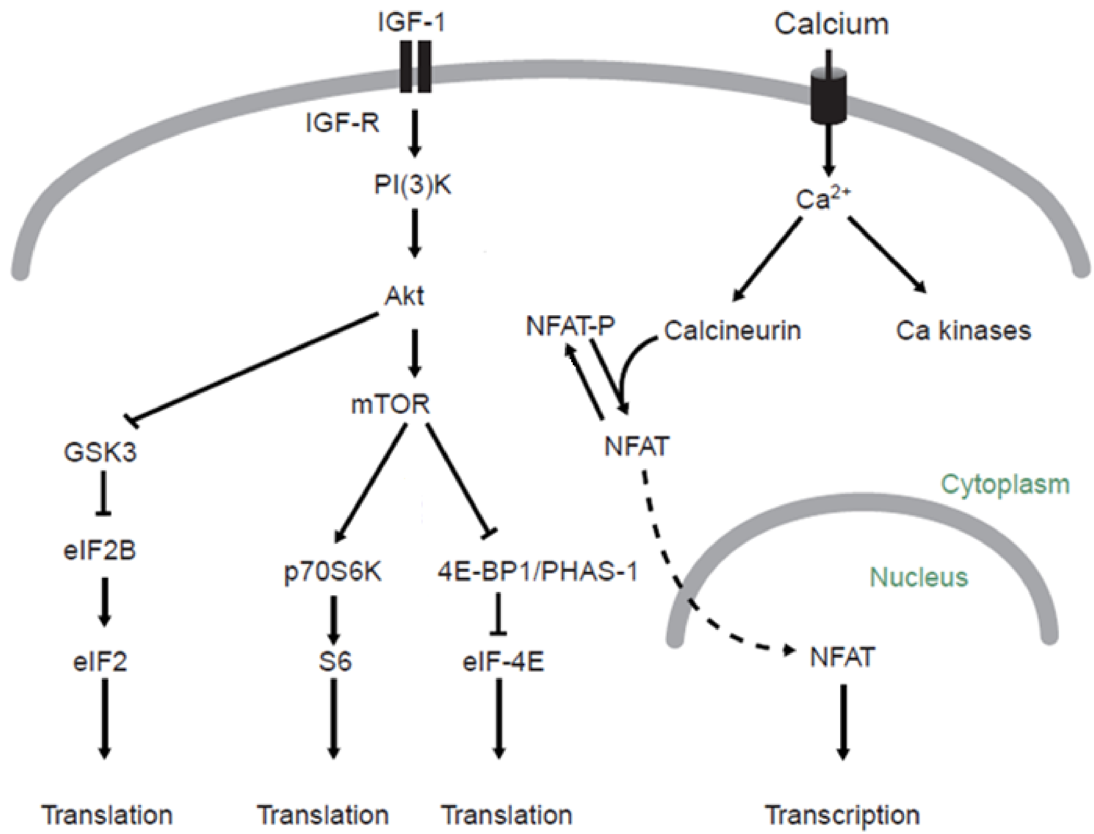


Figure 3. Potential signaling network downstream of agrin

Based on literature, agrin can potentially stimulate these pathways, leading to translational and transcriptional upregulation of genes (modified from **Rommel et al., 2001)**.

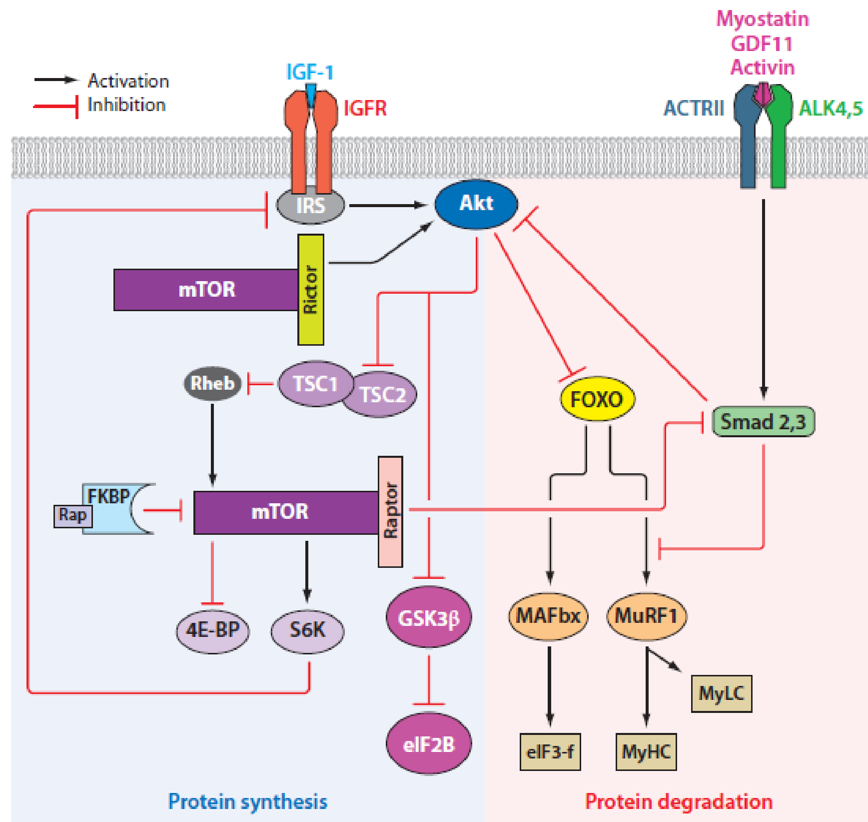


Figure 4. Schematic of hypertrophic and atrophic pathways regulating skeletal muscle growth

IGF-1 and MSTN are the two best characterized pathways that regulate skeletal muscle growth. Akt is the focal point of both pathways. Stimulation by IGF-1 leads to the phosphorylation of Akt which promotes protein synthesis, whereas stimulation by MSTN leads to the dephosphorylation of Akt which promotes protein degradation (from **Ruegg and Glass, 2011)**.

MATERIALS AND METHODS

Reagents

C2C12 cells were treated on Day 0 (Cn model) or Day 2 (mTOR model) of differentiation with Cyclosporin A (CsA; 2 µM; Sandimmune), FK506 (200 nM in dimethylsulphoxide (DMSO)); Enzo Life Science), R3-IGF-1 (10 ng/mL in 10 mM HCl; Sigma), rapamycin (20 ng/mL in DMSO; Calbiochem), or recombinant agrin (1 nM in 1XPBS; R&D Systems). CsA and FK506 were applied to cells at Day 0 of differentiation. Rapamycin and IGF-1 were applied on Day 2 of differentiation. Recombinant agrin was added either at Day 0 or Day 5 of differentiation, depending on the model of growth used for the experiment.

Cell culture

C2C12 cells (gift from Dr. Basil Petrof, McGill University, Montreal, Canada) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine, 1% penicillin and streptomycin under 8% CO_2 at 37°C. Once grown to confluency (approximately 2 days) they were switched to DMEM supplemented with either 2% or 5% horse serum, and 1% penicillin and streptomycin to differentiate the cells. Horse serum induces differentiation by withdrawing the cells from their proliferation phase due to low concentration of growth factors (Levy, 1980). For microscopy analysis, cells were cultured on cover slips and treated with 1.0 nM recombinant agrin for 5 days in differentiation (horse serum) media. For RNA or protein analysis, cells were pelleted after 4 or 5 days of differentiation. Cell culture medium was purchased from Invitrogen.

Myotube analysis

For all treatments, micrographs were taken by capturing three fields at random, and all myotubes in the field were measured for several physiological markers. Myotube diameter was done by taking the average of five measurements along the length of the myotube. Myotube number was determined by counting all myotubes visible in the field and dividing by the area of the image

(myotubes/mm^2). Myotube area was determined by measuring the percentage of the field covered by myotubes. Images were captured using a Nikon Eclipse TS100 microscope with a Nikon Digital Sight camera attachment. The myotubes were identified by tracing their outline and analyzed using ImagePro Plus version 6.3 software (Olympus).

Cell viability assay

To determine cell viability, Day 5 myotubes were washed with Phosphate Buffered Saline (PBS), submerged in 0.2 ml of 0.4% Trypan blue stain (T8154, Sigma), and then incubated at room temperature for 5 min. The dye was decanted then images were captured using a Nikon Eclipse TS100 microscope with a Nikon Digital Sight camera attachment. Dead myotubes stained blue. Cells were counted using a haemocytometer (3120, Hausser Scientific).

Immunofluorescent imaging

Myotubes were grown on a coverslip then fixed in 4 % paraformaldehyde and washed with 1X PBS two times for five minutes each. To label AChR, samples were then incubated in rhodamine-conjugated bungarotoxin (T0195, Sigma) for one hour at room temperature. Samples were washed six times with 1X PBS, dried and mounted on a slide with Vectashield containing 4',6-DiAmidino-2-PhenylIndole (DAPI; Vector Laboratories Inc.) to label nuclei, and sealed with nail polish. Images were captured at 20X magnification using an Olympus BX-60 fluorescence microscope attached with a Retiga SRV camera (Qimaging). Acetylcholine receptor clusters and nuclei were quantified using ImagePro Plus version 6.3 software (Olympus).

RNA extraction and real time quantitative-PCR (qPCR)

Approximately 500,000 cells, determined using a haemocytometer (3120, Hausser Scientific) were scraped and pelleted at 3,000 x g for 5 min at 4°C. To extract RNA from a homogenate, cell pellets were vortexed in a solution made up of 4 M guanidinium thiocyanate (Sigma Aldrich), 25 mM sodium citrate, 0.5% (v/v) N-laurylsarcosine (Sigma Aldrich) and 0.1 M 2-mercaptoethanol (Bioshop), followed by adding 0.2 M sodium acetate (pH 4.0), vortexing, then extracting

proteins from the aqueous phase with 1 vol. phenol (Sigma Aldrich) and 0.1 vol. 24:1 chloroform:isoamyl alcohol. Samples were cooled on ice for 15 minutes, and then centrifuged at 15,000 x g for 10 minutes at 4 ºC. Afterwards, two volumes of 99% ethanol were added to the aqueous phase, which was then vortexed and centrifuged again at 15,000 x g for 10 minutes at 4 ºC. The supernatant was decanted; RNA in the pellet was washed with 500 µl of 70 % ethanol and centrifuged at 15,000 x g for 10 minutes at 4 ºC. Ethanol was again decanted and the RNA pellet was left to dry and suspended in 50 µl of RNAse-free H_2O (Bioshop) with subsequent vortexing and heating at 70 ºC. To test RNA integrity, RNA concentration was determined using an photometer (Eppendorf) at A_{260nm} and 1 µg of RNA was mixed with 2:1 formamide:ethidium bromide, formaldehyde (Sigma Aldrich), 10X 3-(N-MOrpholino) PropaneSulfonic acid (MOPS; pH 7.0) and bromophenol blue, heated at 65 ºC for 10 minutes and loaded in a 1.5 % agarose gel containing 1X MOPS and formaldehyde. The rRNA bands: 5S, 18S and 28S were visualized to indicate valid RNA integrity.

To synthesis cDNA, reverse transcriptase-PCR was performed by combining 1 µg of RNA, ultrapure water, for a final volume of 10 µl. The final volume of the RT mixture was 40 µl, which consisted of 0.625 µM random primer hexamers (Invitrogen), 1X RT-buffer (Ambion), 0.5 µM dNTPs (Invitrogen), 40 U of RNase Inhibitor (Ambion) and 100 U of MMLV-RT (Ambion). To amplify transcripts, PCR was conducted using the settings: 15 minutes at 20 ºC, 1 hour at 37 ºC, and 10 minutes at 65 ºC for one cycle and using an S1000 Thermal Cycler (Bio-Rad). As a negative control, RT samples were duplicated in the absence of MMLV-RT. Resulting cDNA was stored at -20 ºC until used for qPCR analysis.

To amplify cDNA transcripts, quantitative PCR was performed using gene specific primers together with proper reference genes for quantification (CFX96 Real-Time System, Bio-Rad). Relative quantities were then normalized by the Real-time System software to the relative quantities of RPL13 and TBP housekeeping genes. The primers sequences used for qPCR are listed in Table 1.

Protein extraction and immunoblotting

To lyse the cells and expose their proteins for isolation, 500,000 harvested cells, determined using a haemocytometer (3120, Hausser Scientific), were mixed in 1X RadioImmunoPrecipitation Assay (RIPA) buffer solution (Sigma) for one minute consisting of 1X PBS, 1% Igepal, 0.5% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulfate (SDS), 0.001 M Sodium Orthovanadate, 0.01 M Sodium Fluoride, 0.01 mg/ml Aprotinin, 0.01 mg/ml Leupeptin and 1 mM Phenylmethanesulfonyl fluoride. Homogenates were centrifuged at 13,000 x g for 10 minutes and the supernatant layers were collected. Protein concentrations were measured using Bradford assay and measuring their absorbance at A_{595} compared to a standard curve. The whole cell protein was stored at -80 ºC until used for immunoblot anaylsis.

Samples (30 µg) of protein were loaded on an SDS polyacrylamide gel consisting of a 5 % w/v stacking gel composed of 3.9 % acrylamide (Sigma Aldrich), 0.125 M Tris (pH 6.8), 0.1 % SDS, 0.06 % ammonium persulfate and 0.14% Tetramethylethylenediamine (TEMED) (Bioshop), and a 10 % w/v resolving gel composed of 9.9 % acrylamide, 0.375 M Tris (pH 8.8), 0.1 % SDS, 0.06 % ammonium persulfate and 0.25 % TEMED. Samples underwent electrophoresis at 50 V until all of the protein enter the stacking gel then the voltage was increased to 120 V until the protein sizes of interest were visibly separated using the Amersham Full-Range Rainbow Molecular Weight Markers (GE Healthcare Bio-Sciences Corp). Proteins were transferred to a PVDF membrane (Millipore) at 100 V for 100 min, followed by blocking in 3-5 % bovine serum albumin in 0.1 % Tween/Tris Buffered Saline (T/TBS) for one hour. Antibodies (Table 2) were added to membranes based on the manufacturer's (Cell Signaling and Santa Cruz) recommendation: After the incubation period, membranes were washed three times with 0.1 % T/TBS and incubated with secondary antibody coupled to horseradish peroxidase for one hour at room temperature (see Table 2). Membranes were washed three times with 0.1 % T/TBS, developed with enhanced chemi-luminescence reagents (Millipore), and were imaged using the Alpha Innotec Fluorchem system (Cell Biosciences).

Statistical analysis and data presentation

Data was analyzed by ANOVA using SPSS statistics software version 22.0. Multiple comparisons of means were performed with one-way ANOVA followed by a post-hoc test of LSD, while the interactions between experimental conditions were analyzed by using Two Way ANOVA. Only P values < 0.05 were considered significant.

Figures were prepared using Microsoft Excel 2007. Standard error means (S.E.M.) are exclusively shown. Average sample size is n=3. Immunofluorescent images were traced using ImagePro. Modification of immunofluorescent images into greyscale was done using Microsoft Powerpoint 2007. All images had their brightness and contrast increased to increase the visibility of myotubes.

Table 1. Primers used for quantitative real-time PCR

Gene	Forward Primer	Reverse Primer	Size (bp)
Atrogin	5'-aaccgggaggccagctaaagaaca-3'	5'-tgggcctacagaacagacagtgc-3'	288
FoxO1	5'-caggagaagctcccaagtgact-3'	5'-aggaggggtgaagggcatctttggactgc-3'	146
FoxO3a	5'-atcgcctcctggcgggctta-3'	5'-acggcggtgctagcctgaga-3'	297
MEF2c[1]	5'- aggatcaccggaacgaattccact-3'	5'-gcatgcgcttgactgaaggactt-3'	147
MEF2d[2]	5'-cgagatcgcgctcatcatctt-3'	5'-agccgttgaaacccttcttcc-3'	164
MuRF1	5'-gagaacctggagaagcagct-3'	5'-ccgcggttggtccagtag-3'	146
Myf5[3]	5'-ccacctccaactgctctgat-3'	5'-gcaatccaagctggataagg-3'	143
Myogenin	5'-ggaagtctgtgtcggtggac-3'	5'-cgctgcgcaggatctccac-3'	150
Myoglobin	5'-catggttgcaccgtgctcacag-3'	5'-gagcccatggctcagccctg-3'	285
Pax7[4]	5'-tctccaagattctgtgccgat-3'	5'-cggggttctctctcttatactcc-3'	132
RCAN1.4	5'-aaggaacctccagcttgggct-3'	5'-ccctggtctcactttcgctg-3'	160
RPL13	5'-aaggtggtggtcgtacgctgtg-3'	5'-gcgccagaaaatgcggctgg-3'	153
TBP	5'-caccaatgactcctatgacc-3'	5'-gtttacagccaagattcacg-3'	111
TnIs	5'-tgctgaagagcctgatgcta-3'	5'-ggcatggagctctcggcaca-3'	164
Utrophin A	5'-acgaattcagtgacatcattaagtcc-3'	5'-atccatttggtaaaggttttcttctg-3'	75

1. Steffens et al., 2011
2. Primer bank, http://pga.mgh.harvard.edu/primerbank/, ID 19526812a1
3. Kemaladewi et al., 2012
4. Primer bank, http://pga.mgh.harvard.edu/primerbank/, ID 34328055a1

Table 2. Antibodies used for immunoblotting

Protein	Supplier	Size of target protein (kDa)	1° Antibody	2° Antibody
p-GSK3β	Cell Signaling #9323	46	1:3000 in 3% BSA	1:3000 in 3% BSA (anti-rabbit)
Total GSK3β	Cell Signaling #9315	46	1:3000 in 3% BSA	1:3000 in 3% BSA (anti-rabbit)
p-p70S6K	Cell Signaling #9206	70	1:1000 in 3% BSA	1:5000 in 5% BSA (anti-mouse)
Total p70S6K	Santa Cruz sc-230	70	1:1000 in 3% BSA	1:2000 in 5% BSA (anti-rabbit)

RESULTS

Agrin promotes incorporation of nuclei into myotubes

In this study, I hypothesized that the addition of a recombinant C-terminal half of agrin ($AGR_{3,4,8}$) to cultured C2C12 skeletal muscle cells would be sufficient to promote growth. To test this hypothesis, I verified that AGR was biologically active by examining the effect on AChR clustering by immunofluorescence using rhodamine-conjugated α-bungarotoxin (Fig. 5a). AGR-treated cells are known to have an increase of AChR clusters, indicative of NMJs, compared to non-treated cells (Ferns et al., 1993; Gramolini et al., 1998). I observed similar results when treating cells with 1 nM AGR (Fig. 5b). However, while counting the AChR clusters, I also observed that the AGR-treated myotubes were larger than non-treated cells. Therefore, to quantify myotube growth, I stained nuclei using DAPI immunofluorescence (Fig. 5c), as "Nuclei per myotube" is considered a measure of growth (Rommel et al., 2001). We observed a significant 1.8-fold increase (P = 0.004) in the number of nuclei incorporated per myotube present in AGR-treated cells compared to non-treated cells.

Agrin promotes growth via hyperplasia and hypertrophy

After discovering that AGR treatment increases myotube growth, I next sought to determine which second messenger signaling pathways were involved. Since Cn/NFAT signaling is critical in the early stages of growth and development of skeletal muscle cells (Michel et al., 2004), I treated the samples at Day 0 with 1 nM AGR, 2 μM CsA, and 200 nM FK506 (Fig. 6a). This experimental model of growth used in this study is termed the "Cn model". The latter two are Cn inhibitors that act by binding to cyclophillin A and FK506 binding protein 12 (FKBP12), respectively. Both drugs must be used to determine whether the effects are truly due to Cn inhibition since the CsA/cyclophillin A complex targets various other proteins, whereas the FK506/FKBP12 complex is specific to Cn in skeletal muscles. To assess possible toxicity caused by drug treatment, I measured the viability of the C2C12 cells by staining them with 0.4% trypan blue (Fig. 6b). The proportion of stained (dead) cells was below 3% in every group, indicating that the treatments were not toxic. Although a significant increase in non-viable

cells was observed with AGR+CsA and AGR+FK506 samples, it was not drastic enough to physiologically compromise the health of the cells. I then determined effects of treatments on myotube growth by quantifying myotube number and size: Number, an indicator of hyperplasia, was assessed by counting the number of myotubes per mm^2, and size, an indicator of hypertrophy, was assessed by measuring myotube diameter and area. In the presence or absence of CsA or FK506, AGR treatment increased the number of myotubes (Fig. 6c). Although no changes in myotube diameter were observed in any of the samples (Fig. 6d), I observed an increase in myotube area with AGR treatment. However, unlike myotube number, effects of AGR on area were blocked by the Cn inhibitors (Fig. 6e). Together, these preliminary results suggest that AGR induces hyperplasia using a mechanism that does not require Cn, but possibly stimulates hypertrophy using the Cn/NFAT signalling pathway.

Gene analysis of myotubes under calcineurin model of growth

From the results above, it is clear that AGR can promote myotube growth, and that it may require Cn/NFAT signaling for hypertrophy. Thus, I next determined if Cn/NFAT signaling is triggered by AGR treatment by analyzing the mRNA (Fig. 7a, see Table 3 for a summary of results) of genes that are up- or down- regulated by NFAT to mediate myotube hypertrophy. Although CsA and FK506 caused an expected decrease in transcript levels of RCAN1.4, a direct gene target of Cn/NFAT signaling, AGR treatment had no effect on mRNA levels. Similarly, transcription of Utrophin A, TnIs, and myoglobin, other direct targets of Cn/NFAT-signaling, was not affected by AGR. The difference between those three genes and RCAN1.4, however, is that they can also be regulated by other transcription factors. Myoglobin, important for the transport and storage of oxygen, and TnIs, a contractile protein found in slow muscle fibres, can both by upregulated by signaling pathways that contribute to the slow fibre gene program, a program which converts the muscle into a more oxidative state, such as MAPK, AMPK, and CaMK (Ljubicic et al., 2011).

MEF2c and MEF2d are other targets of the program; however, Cn has no influence on their transcription. These data suggest that AGR induces hypertrophy using a signaling pathway that is parallel to Cn/NFAT. These two genes, along with myogenin and Pax7, are also important

transcription factors that regulate myogenesis, the process by which a myoblast differentiated into a myotube (Horsley and Pavlath, 2004). All four genes were upregulated with AGR treatment (Fig. 7b). This indicates that AGR triggers an unknown second messenger that influences myogenic signaling pathways.

To determine, if some of the results stem from possible crosstalk between these pathways, I next examined the effect of AGR on GSK3β phosphorylation. A target of many kinases including Akt, phosphorylation of GSK3β induces export of NFAT from of the nucleus, to impede downstream signaling. Surprisingly, AGR treatment significantly lowered the activity ratio of GSK3β protein, in the presence or absence of Cn inhibitors. As GSK3β is a target of many kinases, and thus a hub for crosstalk, this data suggests that AGR triggers a signaling pathway that infringes on the Cn/NFAT pathway at the level of NFAT-mediated transcription to help trigger hypertrophy.

Agrin has less of an effect on growth when applied at later stages of myotube development

Since positive effects on growth have been observed when AGR was applied early in myotube development (Day 0; Fig. 5-7), I wanted to examine whether it had an effect when applied later in its development. IGF-1, can act via many signaling pathways, one of which is the Akt/mTOR signaling cascade, one of the pathways that regulate hypertrophy (Musaro et al., 2001). The pathway is primarily active once the myotubes have already been formed and need to grow in size. To study the pathway, along with the effect of agrin at the later stages of development, on Day 2 I begun the supplementation in the media of 1 nM AGR, 10 nM IGF-1 (positive control), and 20 nM rapamycin (RAP; negative control). I termed this experimental model of growth the "mTOR model".

Again, I confirmed that drug treatment had little effect on cell viability. As shown previously (Fig. 6b), the amount of non-viable cells present was less than 3.0% of the total cells, indicating that the drugs had no adverse effects (Fig. 8b). As previously reported (Rommel et al., 2001), IGF-1 and RAP significantly increased and decreased, respectively, myotube number, diameter, and area (Fig 8c-e). Furthermore, RAP blocked effects of IGF-1 as expected. Although

AGR did not significantly increase myotube number and area, an upward trend was still observed. Interestingly, RAP also blocked the effect of AGR on myotube number and area, suggesting that AGR-mediated myotube growth requires active mTOR when applied at later stages of development.

Effect of blocking mTOR on agrin-mediated changes in transcription

Because blocking mTOR impaired AGR-mediated growth, I next wanted to determine if RAP also blocked effects of AGR on transcription of genes regulating myotube growth. Because mTOR is also a well-known mediator of atrophy, I decided to focus on studying the transcriptional levels of genes involved in this process. FoxO1 is a transcription factor that regulates MuRF1 mRNA expression, and FoxO3a is a transcription factor that regulates atrogin mRNA transcription. In both cases, the downstream targets are components of the ubiquitin-proteasome system responsible for protein degradation (Glass, 2003). Atrogin and MuRF1 are both down regulated in the presence of IGF-1, as well as with AGR treatment, consistent with the observation that both promote myotube growth (Fig. 9a). Again, I examined the extent of p70S6K phosphorylation (Fig. 9b) and confirmed an increase in phosphorylation in IGF-1 treated cells compared to CTL and a decrease in RAP samples, as expected. An increase was also observed in AGR samples which indicate that AGR does influence the Akt/mTOR pathway when applied at later stages of development. IGF+RAP and AGR+RAP increased activity in p70S6K compared to RAP samples agrees with the observations thus far in this thesis that AGR can act on multiple pathways.

Next, I determined if RAP could block effects of AGR on gene expression that correlates with myotube growth (Fig. 10). Pax7 is a positive regulator of satellite cell activation, one of the phases in myogenesis (Buckingham et al., 2003). Interestingly, its relative transcript level is significantly decreased with AGR treatment, likewise for Myf5, an important gene in the determination step of myogenesis, where the stem cell (or satellite cell) develops into a muscle cell (Buckingham et al., 2003). Altogether, changes in these two genes seem to indicate that AGR does not positively promote myogenesis like it did when applied at the early stages of

development. This could explain why no significance was found for morphological markers in Fig. 8.

The phosphorylation ratio of GSK3β was measured and IGF treatment, with or without RAP, was significantly increased. Since mTOR does not interact with GSK3β, it makes sense that the activity was not changed with the inhibitor present. However, AGR treatment with mTOR inhibitor is significantly less than without the inhibitor (Fig. 10b). This result could signify that the treatment of AGR and RAP on those cells may have had a compounding effect on the activation of the protein.

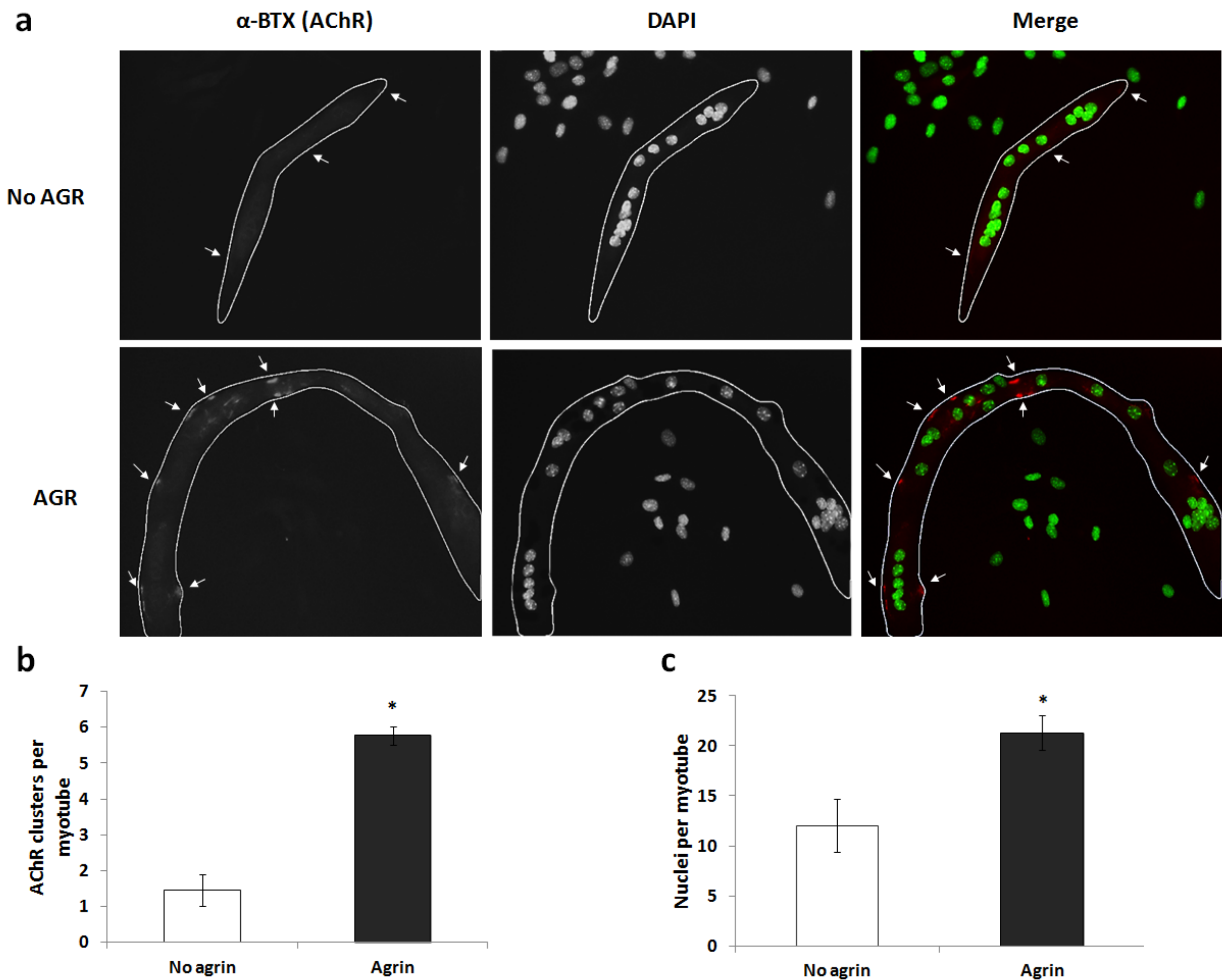
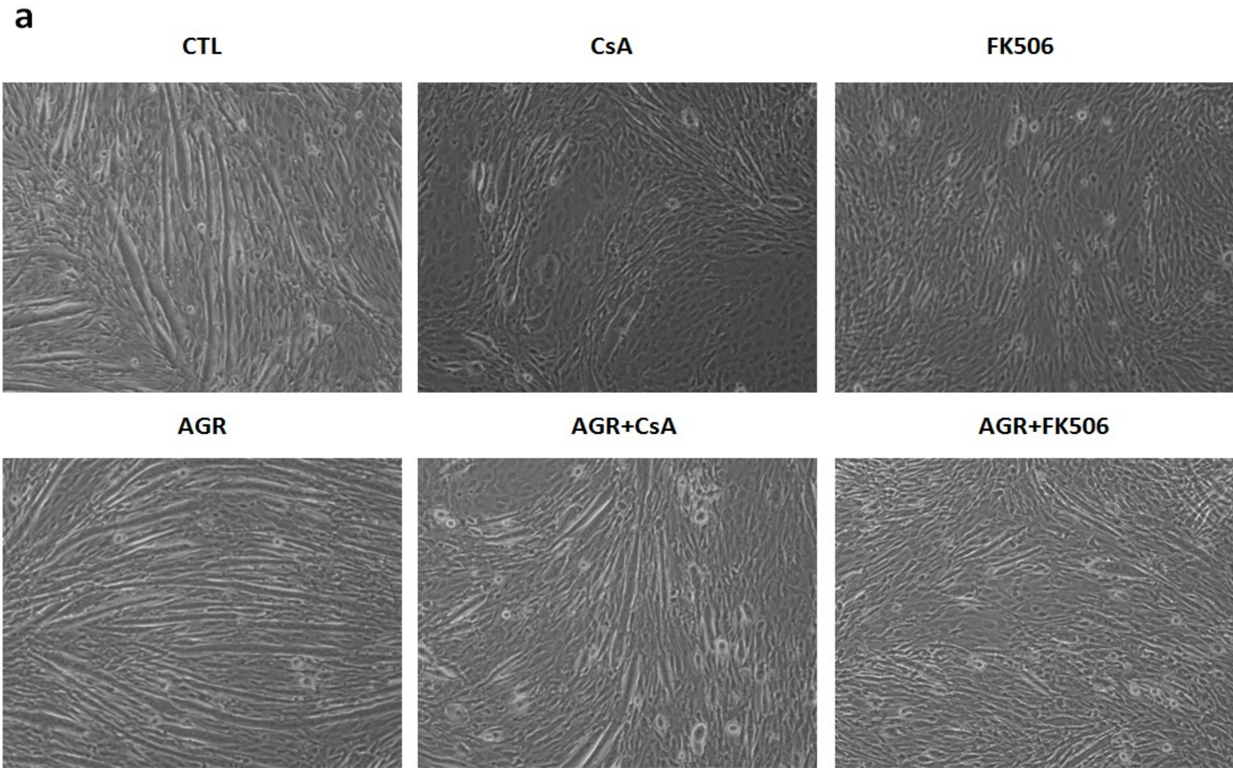
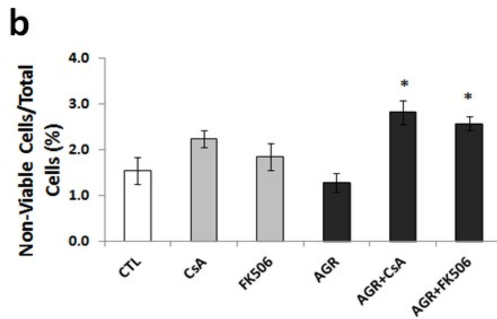
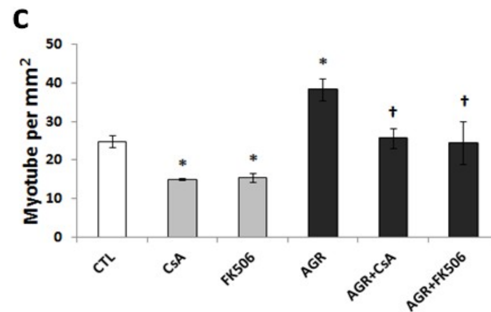
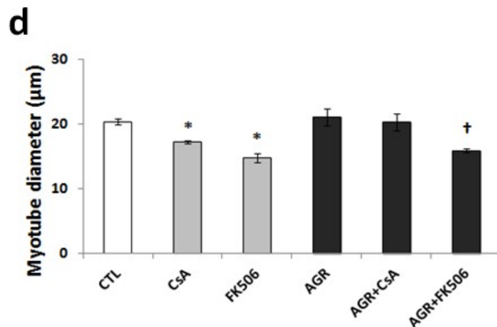


Figure 5. Agrin promotes acetylcholine receptor clustering and myotube growth
(a) Representative immunofluorescent images C2C12 myotubes treated with 1 nM recombinant agrin for 5 days. AChR (red) and nuclei (green) are stained. Quantification of AChR clustering **(b)** and nuclei per myotube **(c)** are shown (n=3). *Indicates statistical significance when compared to control (no AGR) ($P < 0.05$). Means ± SEM are shown.









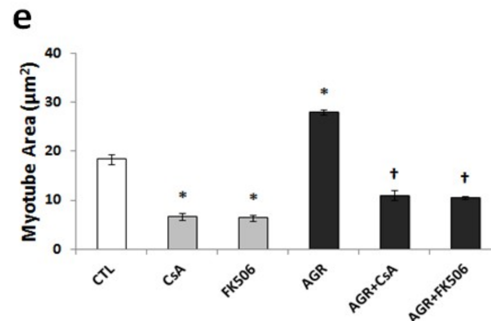


Figure 6. Agrin requires calcineurin for hypertrophy but not hyperplasia
(**a**) Representative light micrographs of C2C12 myotubes treated with 1nM AGR, 2µM CsA, and/or 200nM FK506. **(b-e)** Quantifications of morphological growth markers derived from the images similar to those shown in (a). * indicates statistical significance when compared to CTL, † when compared to AGR ($P < 0.05$). Means ± SEM are shown.

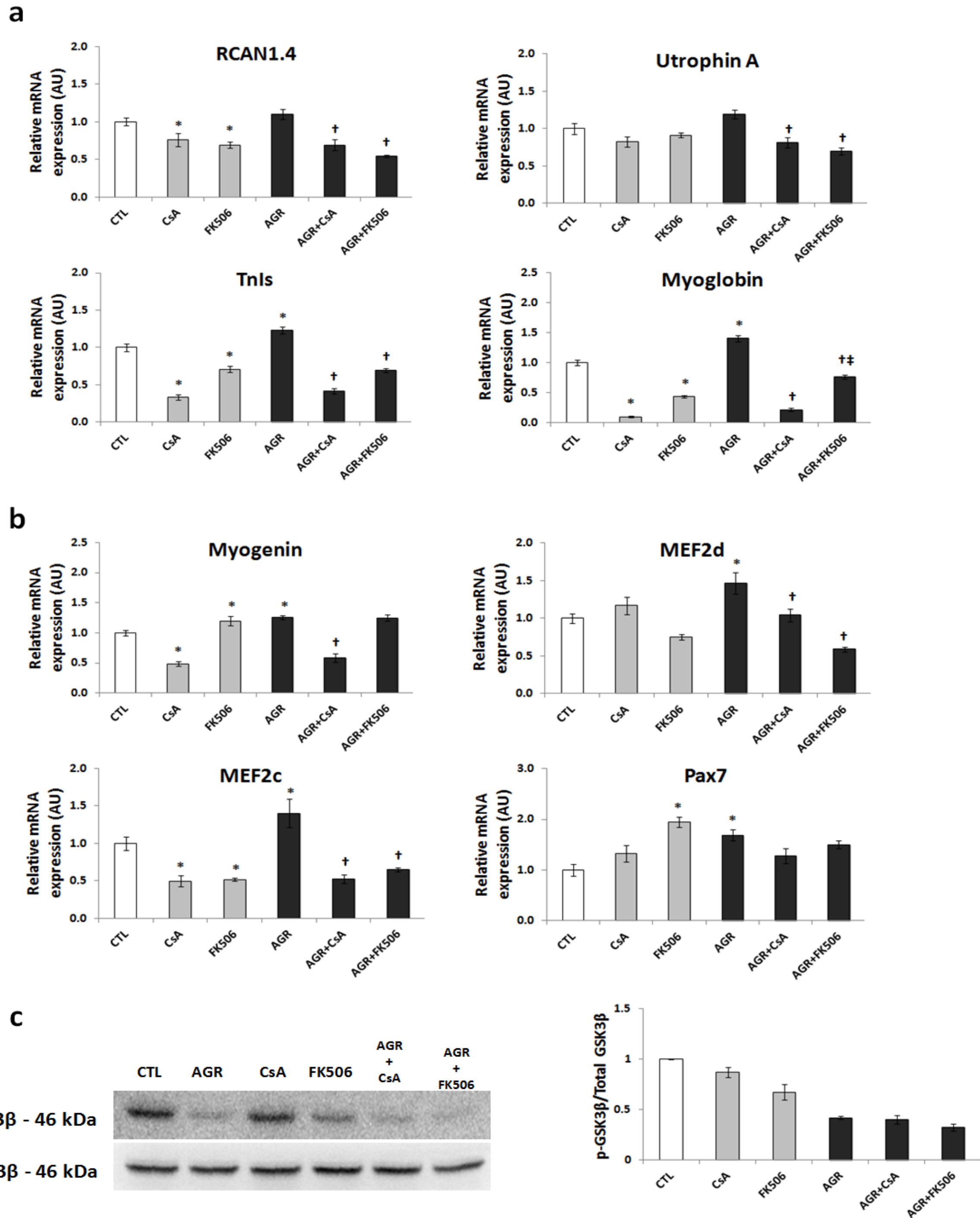


Figure 7. Agrin influences myogenic and hypertrophic signaling pathways independent of calcineurin/NFAT signaling

Changes in relative mRNA expression levels using qPCR analysis for various genes related to Cn/NFAT signaling **(a)** and myogenic signaling **(b)** normalized to RPL13 and TBP (n=3). **(c)** Representative immunoblot and changes in relative phosphorylated protein expression levels using immunoblot analysis for GSK3β (n=3). * indicates statistical significance when compared to CTL, † when compared to AGR ($P < 0.05$). Means ± SEM are shown.

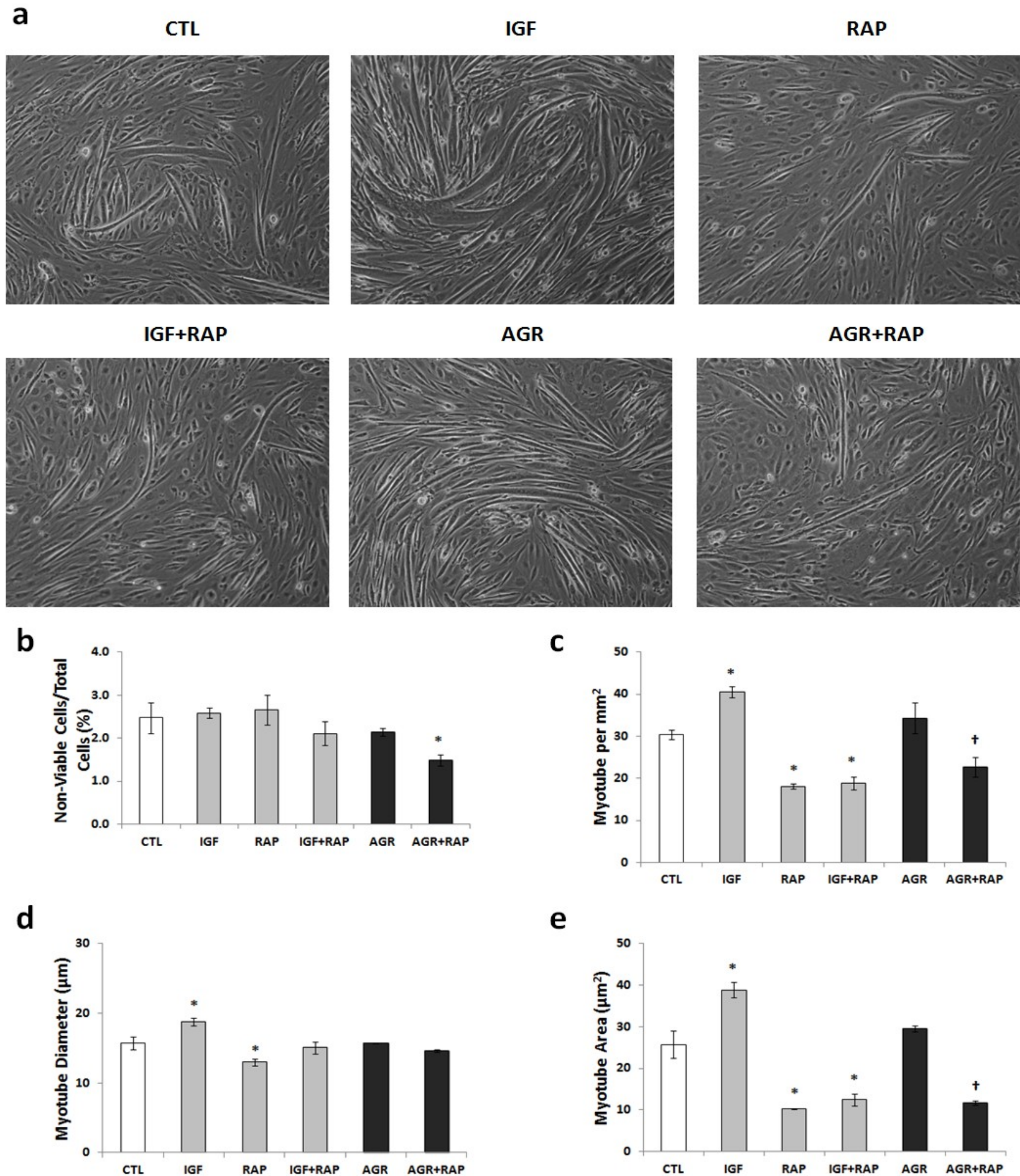


Figure 8. Agrin has a less prominent effect on growth in the mTOR model
(a) Representative images of C2C12 myotubes treated with 1 nM AGR, 10 nM IGF-1, and 20 nM RAP. **(b-e)** Quantifications of morphological growth markers derived from the images in (a). * indicates statistical significance when compared to CTL, † when compared to AGR ($P < 0.05$). Means ± SEM are shown.

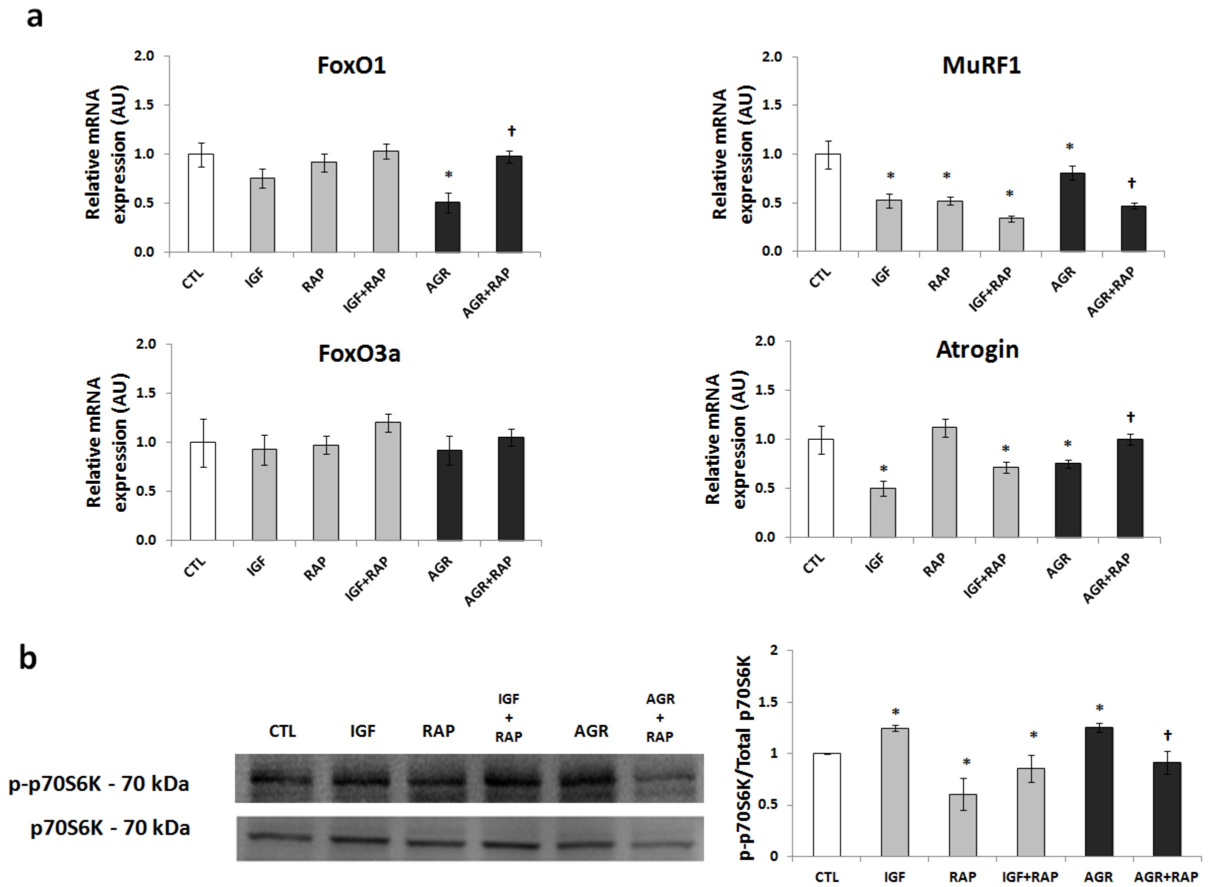


Figure 9. Agrin decreases atrophy and promotes protein synthesis via mTOR
(a) Changes in relative mRNA expression levels using qPCR analysis for atrophy-related genes normalized to RPL13 and TBP (n=3). **(b)** Representative immunoblot and changes in relative phosphorylated protein expression levels using immunoblot analysis for p70S6K (n=3). * indicates statistical significance when compared to CTL, † when compared to AGR ($p < 0.05$). Means ± SEM are shown.

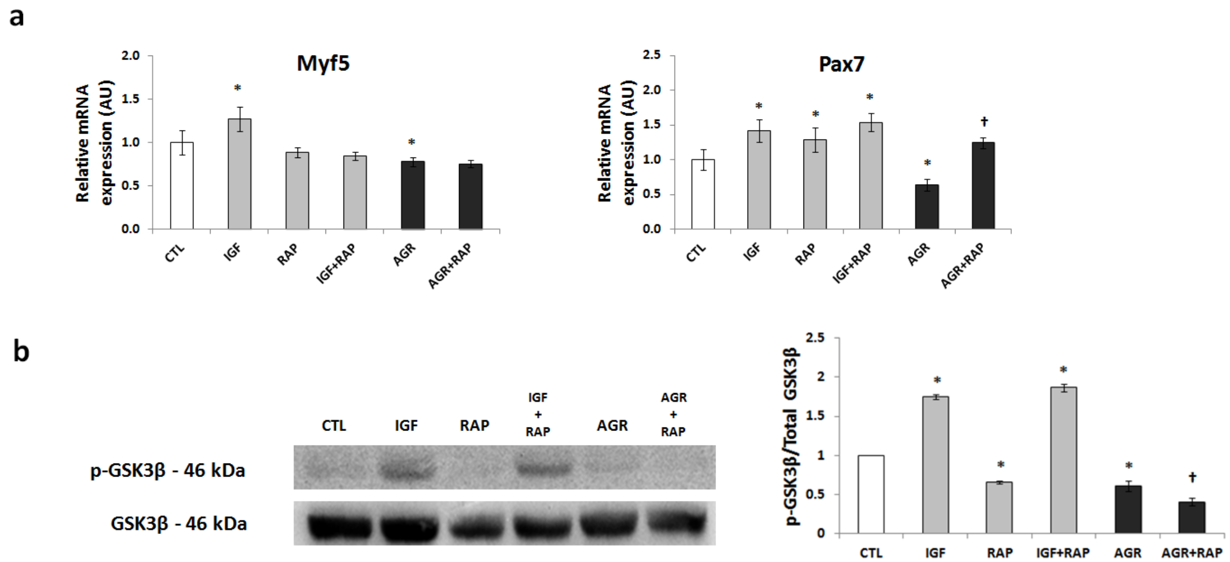


Figure 10. Agrin does not positively regulate myogenesis in the mTOR model
(a) Changes in relative mRNA expression levels using qPCR analysis for myogenic factors normalized to RPL13 and TBP (n=3). **(b)** Representative immunoblot and changes in relative phosphorylated protein expression levels using immunoblot analysis for GSK3β (n=3). * indicates statistical significance when compared to CTL, † when compared to AGR ($p < 0.05$). Means ± SEM are shown.

Table 3. Summary of effects of agrin on signaling targets

Application of AGR	Process	Function	Gene	Effect on gene transcription
Early	Myogenesis	Transcription factor	MEF2c	↑
			MEF2d	↑
			Pax7	↑
			Myogenin	↑
	Mitochondrial biogenesis	Transcription factor	PGC-1α	↑
	Contraction	Contractile protein	TnIs	↑
	Oxygen transport	Transports oxygen to muscles	Myoglobin	↑
	Cn inhibition	Binds catalytic domain of CnA	RCAN1.4	-
	Structural stability	Binds cytoskeleton to plasma membrane	Utrophin A	-
	Apoptosis	Transcription factor	FoxO3a	↑
			Smad2	↑
	Protein degradation	E3 ligase	MuRF1	↑
Late	Apoptosis	Transcription factor	FoxO1	↓
			FoxO3a	↓
	Protein degradation	E3 ligase	MuRF1	↓
			Atrogin	↓
	Myogenesis	Transcription factor	Myf5	↓
			Pax7	↓

DISCUSSION

The work presented in this study demonstrates the role of agrin in skeletal muscle growth. I found that AGR can promote growth via hypertrophy and hyperplasia, while also upregulating many myogenic factors, thus, also promoting the fusion of myoblasts into myotubes. This confirms that AGR has a strong role in the early stages of myotube development. The exact mechanism by which AGR-mediated growth acts was not elucidated in this study, however, I found evidence that it is a pathway that shares common targets to Cn/NFAT signaling. A schematic has been drawn to help organize the data presented in this study (Fig. 11).

Agrin promotes growth in C2C12 cells

Upon analysis of fluorescent staining of nuclei with DAPI, a trend was observed in which the cells treated with AGR had many more nuclei. AGR treatment on skeletal muscle cells affected their growth by promoting the fusion of myoblasts into myotubes, an indicator of myogenesis upregulation (Mancini et al., 2011). The phase of growth that it affected was their differentiation since there were more myotubes with increased nuclei compared to CTL. An increase in the differentiation of cells leads to more myotubes being formed which is a form of growth called hyperplasia. Since AGR treated cells not only grew in number but also size, it was confirmed that AGR promoted growth not only through hyperplasia, but also hypertrophy.

The connection between myogenic factors and agrin in skeletal muscles has been documented before (Ball, 2013). Although they were studying the effects of myogenin and myoD on agrin-mediated AChR clustering, they did not mention any effects on the growth of the cultured myotubes. They found that myogenin and MyoD are downstream of agrin/MuSK signaling. This explains the increase in myogenin mRNA in AGR treated cells I observed, however, the MyoD was not upregulated in my study (data not shown). An explanation for this occurrence is that MyoD acts in the early stages of differentiation when the cells are still primarily myoblasts. As the cells fuse to become myotubes, the presence of MyoD drops off (Ball, 2013). On the other hand, myogenin acts late in the differentiation process, and increases as myotubes form. Since my samples were harvested and analyzed at Day 5 of differentiation,

MyoD transcripts were likely degraded by that time. To investigate this further, cells should be harvested at Day 2 or earlier, and measure MyoD mRNA to observe an upregulation.

Agrin does not interact with the calcineurin/NFAT pathway

The Cn/NFAT pathway is one of interest due to its notable involvement in differentiation of skeletal muscle myoblasts (Friday et al., 2000). CsA and FK506 treated cells gave an insight into how the growth is being regulated. The results show that growth is partially restored in AGR treated cells with Cn inhibitors, indicating that AGR-mediated growth is sufficient to overcome the atrophic state induced by the Cn inhibitors. The fact that growth did not rise to the same extent as without Cn inhibitors could be a matter of the concentrations of agrin or Cn inhibitors used. A future study can be conducted where a higher dosage of AGR is supplied to the cells to observe that fully restores growth in Cn inhibited cells. If not, then it can be concluded that AGR-mediated growth shares components of the Cn/NFAT pathway. Another study can be conducted wherein the concentration of Cn inhibitors is lowered. Although the concentrations used in this thesis were not detrimental to the viability of the cells (Fig. 6b), it is possible that lower concentrations can still be used since a concentration gradient was performed (data not shown) and lower levels of CsA and FK506 were still able to inhibit myotube development. In the end, however, the basis for the partial rescue of Cn inhibited cells by AGR treatment is unknown and must be looked at further with future experiments.

The mRNA targets shown in Fig. 7a are downstream targets of Cn signaling as well as other signaling pathways that are all part of the slow fibre gene program. The oxidative, or "slow", fibre gene program is a set of signaling pathways (MAPKs, Cn, CaMKs, AMPK pathways) that lead to more oxidative and energy efficient skeletal muscle fibres. Slow fibres also contain a higher percentage of mitochondria per cell compared their fast fibre counterparts. The increase in relative mRNA in AGR-treated cells is significant across most targets, indicating that Cn has a role to play in AGR-related growth. One gene in particular, MEF2c, is an important factor in myoblast fusion into myotubes and differentiation (Baylies and Michelson, 2001). The up regulation in AGR cells compared to CTL cells of the expression of MEF2c suggests that it may be responsible for the increased differentiation and hyperplasia observed. However,

RCAN1.4, a gene under direct transcriptional control from Cn is not significantly increased. Neither is utrophin A which is also heavily regulated by Cn signaling. Furthermore, we can see that the cells treated with CsA and FK506 do not behave identically even though they are both Cn inhibitors. As mentioned earlier, this is due to the fact that FK506 solely inhibits Cn whereas CsA binds to multiple other targets thus affecting other pathways. Cyclosporin A secondarily targets the mitochondria thus having effects on various related pathways such as MAPKs, CaMKs, and AMPK (Schiaffino et al., 2007). Since mRNA targets analyzed in Fig. 7a such as myoglobin and TnIs, demonstrate a more drastic decrease in relative mRNA levels in AGR+CsA cells compared to AGR+FK506 cells, points to the fact that CsA is also inhibiting all pathways contributing to the slow fibre gene program. Given the fact that RCAN1.4 did not increase with AGR treatment indicates that the addition of AGR to cells did not stimulate Cn signaling. Altogether, the results show that AGR promotes growth via the other pathway(s) that contributes to the slow fibre program. Recent evidence suggest that it might be MAPK pathway (Rimer, 2011). The MAPKs have three pathways in which to act by; the one of interest is the MEK/ERK signaling cascade. It has been found to be agrin treatment at the NMJ, through MuSK signaling, which upregulated that pathway. MEK/ERK cascade has also been implicated in activation of the slow fibre program and promotion of differentiation in skeletal muscle cells. This strongly correlates with the phenotypes observed in my study, indicating that the MEK/ERK pathway might be the major component in the role of agrin in growth.

The results from measuring GSK3β activity by immunoblot analysis did not help determine whether AGR stimulated Cn signaling since the protein is targeted by many other pathways. The results obtained indicate that AGR treatment can influence the activity of GSK3β; however, as we found out from the results in Fig. 7a, AGR is unlikely to stimulate Cn activity. The evidence for AGR affecting GSK3β phosphorylation, without Cn activity, is when the comparison between Cn inhibitors with or without AGR is done. GSK3β activity is lower in every sample with AGR treatment when compared to their non-AGR treated counterparts. Furthermore, if Cn was stimulated, GSK3β activity should have been increased with AGR treatment and not decreased as the results obtained show. It is possible that the lowered activity is due to interactions between AGR and the Wnt signaling pathway, which can affect the phosphorylation levels of GSK3B (Metcalfe and Bienz, 2011). Wnt signaling can bind to the

LRP4/MuSK receptor complex, prompting the dishevelled, Dvl, complex to dephosphorylate GSK3β (Shi et al., 2012). This pathway is also prompted by agrin, since LRP4/MuSK is the same receptor complex used to induce AChR clustering, therefore explaining the decrease in phosphorylation ratio of GSK3β in AGR treated cells.

Agrin is involved in the earlier stages of myotube development

Using the Cn model for cell growth, it did not appear that other major growth pathways, IGF/Akt and MSTN signaling, were stimulated by AGR treatment (data not shown). The mTOR model of cell growth allowed me to explore the later aspect of growth. Using the same morphological markers as Fig. 6, it can be seen that AGR does not stimulate growth to the same extent (Fig. 8). The mTOR model has no significance for myotube number or area in AGR treated cells, whereas using the Cn model did. This implicates the role of agrin in growth in the earlier stages of myotube development. Although AGR had less of an effect on growth in the mTOR model, I was able to conclude its interaction with the Akt/mTOR pathway through the upregulation of p70S6K activity (Fig. 9b).

Concluding remarks

In summary, the results presented in this thesis provide a role for AGR in skeletal muscle growth *in vitro*. The data presented suggest that AGR treatment is best when supplemented during the early phases of myotube development to ensure a more significant growth. There are various limitations of using this *in vitro* model as it does not include any of the other signals that would be released by the neuron, nor does it have blood vessels. Although studying the role of AGR in adult muscle fibers would be best, this allows us to explore its role in an isolated, controlled system. Since AGR plays such a prominent role in myotube differentiation, it is possible that it would only be important during an organism's embryonic phase, while not having much of an effect in adult muscle fibers. There is still much unknown about the new role of agrin in skeletal muscle cells, therefore, it is hard to tell what the implications of effecting its biological concentrations would be for an *in vivo* system, and thus, future experiments must be

done. Agrin would not be the first neurotrophic factor to promote growth in skeletal muscles: brain-derived neurotrophic factor (Clow and Jasmin, 2010) and neurotropin-4 (Funakoshi et al., 1995) are other neurotrophic factors that have been shown to regulate skeletal muscle growth. Although it remains unclear which growth pathway AGR interacts with, the data presented in this thesis suggest that it is likely through MAPKs, CaMKs, and/or AMPK. Given that there are recent studies indicating that AGR interacts with proteins in the MAPK family such as MEK and ERK (Rimer, 2011), it would be vital to explore that pathway in future work.

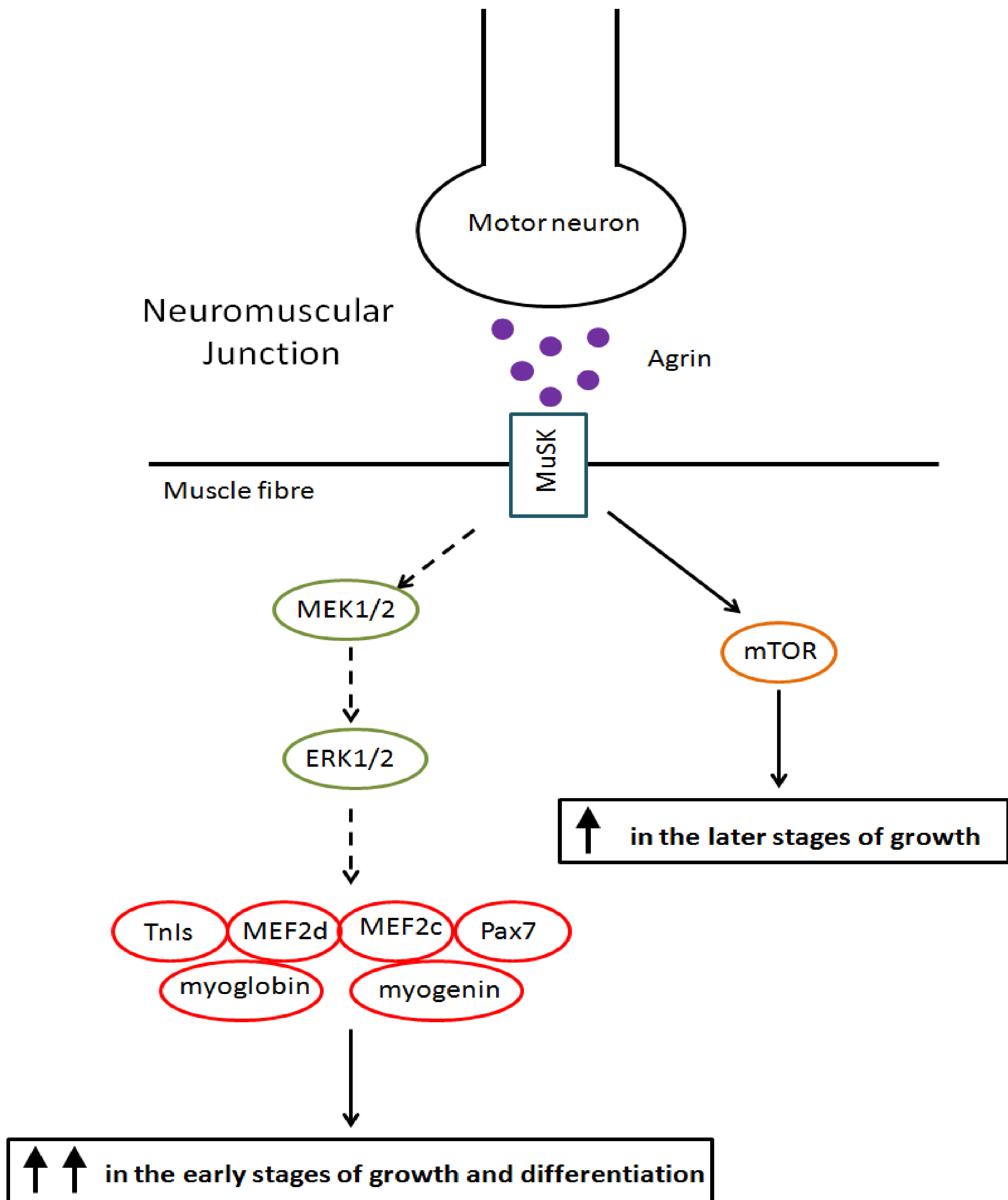


Figure 11: Proposed signaling cascade for agrin-related growth in skeletal muscle cells
Agrin was found to stimulate mTOR at later stages of development which leads to growth. Evidence from literature points to MAPK signaling as the major pathway being used in agrin-related growth, however, that has not been confirmed in this study and must be explored. Dashed lines represent data not studied in this thesis.

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