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► **To cite this version:**

Olivier R Joassard, Guy Bélanger, Jennifer Karmouch, John A Lunde, Anu H Shukla, et al.. HuR mediates changes in the stability of AChR  $\beta$ -Subunit mRNAs after skeletal muscle denervation. *Journal of Neuroscience*, 2015, 35 (31), pp.10949-10962. 10.1523/JNEUROSCI.1043-15.2015 . hal-01837616

**HAL Id: hal-01837616**

**<https://hal.science/hal-01837616>**

Submitted on 7 Jun 2021

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# HuR Mediates Changes in the Stability of AChR $\beta$ -Subunit mRNAs after Skeletal Muscle Denervation

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Acetylcholine receptors (AChRs) are heteromeric membrane proteins essential for neurotransmission at the neuromuscular junction. Previous work showed that muscle denervation increases expression of AChR mRNAs due to transcriptional activation of AChR subunit genes. However, it remains possible that post-transcriptional mechanisms are also involved in controlling the levels of AChR mRNAs following denervation. We examined whether post-transcriptional events indeed regulate AChR  $\beta$ -subunit mRNAs in response to denervation. First, *in vitro* stability assays revealed that the half-life of AChR  $\beta$ -subunit mRNAs was increased in the presence of denervated muscle protein extracts. A bioinformatics analysis revealed the existence of a conserved AU-rich element (ARE) in the 3'-untranslated region (UTR) of AChR  $\beta$ -subunit mRNA. Furthermore, denervation of mouse muscle injected with a luciferase reporter construct containing the AChR  $\beta$ -subunit 3'UTR, caused an increase in luciferase activity. By contrast, mutation of this ARE prevented this increase. We also observed that denervation increased expression of the RNA-binding protein human antigen R (HuR) and induced its translocation to the cytoplasm. Importantly, HuR binds to endogenous AChR  $\beta$ -subunit transcripts in cultured myotubes and *in vivo*, and this binding is increased in denervated versus innervated muscles. Finally, p38 MAPK, a pathway known to activate HuR, was induced following denervation as a result of MKK3/6 activation and a decrease in MKP-1 expression, thereby leading to an increase in the stability of AChR  $\beta$ -subunit transcripts. Together, these results demonstrate the important contribution of post-transcriptional events in regulating AChR  $\beta$ -subunit mRNAs and point toward a central role for HuR in mediating synaptic gene expression.

**Key words:** AChR  $\beta$ -subunit mRNA; HuR; p38 MAPK; skeletal muscle denervation

## Significance Statement

Muscle denervation is a convenient model to examine expression of genes encoding proteins of the neuromuscular junction, especially acetylcholine receptors (AChRs). Despite the accepted model of AChR regulation, which implicates transcriptional mechanisms, it remains plausible that such events cannot fully account for changes in AChR expression following denervation. We show that denervation increases expression of the RNA-binding protein HuR, which in turn, causes an increase in the stability of AChR  $\beta$ -subunit mRNAs in denervated muscle. Our findings demonstrate for the first time the contribution of post-transcriptional events in controlling AChR expression in skeletal muscle, and points toward a central role for HuR in mediating synaptic development while also paving the way for developing RNA-based therapeutics for neuromuscular diseases.

## Introduction

Transduction of the acetylcholine signal at the neuromuscular junction (NMJ) to trigger muscle contraction relies on a high density of

acetylcholine receptors (AChRs) on the surface of the postsynaptic membrane. AChRs are well characterized heteromeric transmembrane proteins made up of four constitutive subunits (2  $\alpha$ , 1  $\beta$ , and 1  $\delta$ ) and one variable  $\gamma$  subunit that switches to  $\epsilon$  during muscle development to form adult AChRs (Mishina et al., 1986; Duclert and

Received March 16, 2015; revised June 2, 2015; accepted June 22, 2015.

Author contributions: O.R.J., G.B., J.K., A.C., C.L., and B.J.J. designed research; O.R.J., G.B., J.A.L., A.H.S., and A.C. performed research; O.R.J., G.B., A.C., and B.J.J. analyzed data; O.R.J. and B.J.J. wrote the paper.

This work was supported by grants from the Association Française contre les Myopathies (AFM; to B.J.J. and C.L.), the Canadian Institutes of Health Research (B.J.J.), the Canadian Space Agency (B.J.J.), the Centre National d'Etudes Spatiales (A.C.), and J.O.R. was supported by a postdoctoral fellowship from AFM. We thank Drs Imed Gallouzi and Alex MacKenzie for providing various reagents, members of the Jasmin laboratory for insightful discussions, and Christine Péladeau for technical assistance.

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.1043-15.2015

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Changeux, 1995; Missias et al., 1996). Over the last 3 decades, considerable efforts have been invested to understand how the highly localized expression of AChRs within the postsynaptic apparatus occurs (Sanes and Lichtman, 2001). Overall, such an accumulation of AChRs may be seen as a two-step process. First, myogenesis is initially marked by a rapid burst of AChR synthesis with the subsequent clustering of these newly synthesized AChRs at sites of nerve-muscle contacts. Second, once the synaptic contacts are established, expression of AChRs becomes restricted to synaptic regions of muscle fibers with active repression in extrasynaptic compartments (Willmann and Fuhrer, 2002). The molecular mechanisms regulating expression of AChRs during myogenesis, synapse formation, and at adult NMJs are well understood and thought to mostly involve transcriptional regulatory pathways (J. Tang et al., 1994; Bessis et al., 1995; Koike et al., 1995; Duclert et al., 1996; Tansey et al., 1996; Altiok et al., 1997; Burden and Yarden, 1997; Fischbach and Rosen, 1997; Sandrock et al., 1997; Schaeffer et al., 1998; Massari and Murre, 2000; Macpherson et al., 2006; H. Tang et al., 2006).

Nerve transection, causing skeletal muscle denervation, has been widely used as a convenient experimental system to also study AChR gene regulation. Thus, skeletal muscle denervation markedly increases the level of  $\alpha$ -,  $\beta$ -,  $\delta$ -, and  $\gamma$ -subunit mRNAs (Evans et al., 1987; Goldman et al., 1988; Witzemann, 1989) to different extent depending on the specific muscle, animal species and AChR subunits considered. Such increases in AChR expression in denervated muscle occurs in extrasynaptic regions of muscle fibers and they have largely been attributed also to increased transcriptional rate resulting in a dramatic increase in AChR synthesis which causes acetylcholine supersensitivity (Lomo and Westgaard, 1975; Merlie et al., 1984; Buonanno and Merlie, 1986; Neville et al., 1998).

Despite the overall model of AChR gene regulation which primarily implicates transcriptional mechanisms, it has been proposed that such molecular events cannot fully account for the changes seen in the levels and localization of synaptic mRNAs in muscle cells (Chakkalakal and Jasmin, 2003) and that therefore, other mechanisms are likely involved and essential in regulating AChR expression. The central objective of the current study was to explore whether post-transcriptional mechanisms, operating at the level of mRNA stability, contribute to the changes in AChR mRNAs seen in denervated muscle. In our work, we also examined the putative involvement of the RNA-binding protein (RBP) human antigen R (HuR) in controlling AChR mRNA stability in skeletal muscle. Given the central role of the AChR  $\beta$ -subunit in agrin-induced cytoskeletal anchoring, AChR aggregation (Friese et al., 2007; Borges et al., 2008), and stabilization of newly form AChR clusters (Rudell and Ferns, 2013), and furthermore, considering the fact that the AChR  $\beta$ -subunit has been shown to be essential for AChR assembly (Quiram et al., 1999), perhaps even constituting a rate-limiting factor in the assembly of pentameric AChRs (Claudio et al., 1989; Saedi et al., 1991), we chose to focus our effort on this specific AChR subunit.

## Materials and Methods

**Animal care.** Seven-week-old female C57BL/6 mice, purchased from Charles River Laboratories, were maintained in the Animal Care and Veterinary Service of the University of Ottawa under a constant 12 h light/dark cycle with food and water *ad libitum*. Experiments were approved by the University of Ottawa Institutional Animal Care and User Committee and were performed in accordance with the established guidelines of the Canadian Council on Animal Care.

**Hindlimb surgical denervation and tissue removal.** For denervation, mice were anesthetized <2–3% isoflurane. The left sciatic nerve was sectioned and, to avoid reinnervation, a 2–3 mm segment was removed.

**Table 1. Oligonucleotide primers used for PCR analysis**

Gene	Primer sequences 5'–3'
AChE (Ache; NM_001290010.1)	Fwd: CCGAGGCTCTCATCAACTGCTG Rev: GGGACCCCGTAACCAACGAAAG
AChR $\beta$ -subunit (Chrb1; NM_009601.4)	Fwd: CATCATCGCTACCCAC Rev: ACGGTCCACAACCATGGC
$\beta$ -Actin (Actb; NM_007393.3)	Fwd: CCCTGTATGCCTCTGGTCTG Rev: ATGGCGTAGGGAGAGCAT
GAPDH (Gapdh; NM_008084.3)	Fwd: GGGGTGAACACGAGAAAT Rev: CCTCCACAATGCCAAGTT
HuR (Elavl1; NM_010485.3)	Fwd: CAGAGGTATCAAAAGATGC Rev: ATCCACTCATGTGATCTAC
Myogenin (Myog; NM_031189.2)	Fwd: TCACATAAGGCTAACCCACG Rev: GGAATTCGAGGCATATTATGA
18S ribosomal RNA (Rn18s; NR_003278.3)	Fwd: CGCCGCTAGAGGTGAATC Rev: CCACTCGGCATCTTTATGG

Primers were designed using Primer 3 software from gene sequences obtained from GenBank. Primer specificity was determined using a BLAST search.

The skin was then closed with sterile clips. The contralateral leg remained innervated and served as a control. Three days after denervation, mice were killed and *tibialis anterior* (TA) muscles were excised and immediately frozen in liquid nitrogen or, embedded in Tissue-Tek OCT Compound (Sakura Finetek) and frozen in melting isopentane precooled with liquid nitrogen. All muscles samples were stored at  $-80^{\circ}\text{C}$  for subsequent analyses.

**RNA isolation and RT-qPCR.** Total RNA was extracted from TA muscles using TriPure isolation reagent (Roche Diagnostics) as per the manufacturer's recommendations. TriPure-extracted RNA was treated for 1 h with DNase I (Invitrogen) to eliminate possible genomic DNA contamination. Real-time quantitative PCR was performed on reverse transcribed RNA using the QuantiTect SYBR Green PCR kit (Qiagen) on an MX3005p real-time PCR system (Stratagene). The selected forward and reverse primer sequences are listed in Table 1. All reactions were performed in duplicate. All mRNAs of interest used the same cycling parameter: the thermal conditions consisted of an initial denaturation step at  $95^{\circ}\text{C}$  for 10 min, followed by 40 cycles of denaturation at  $95^{\circ}\text{C}$  for 15 s, annealing at  $60^{\circ}\text{C}$  for 30 s, and extension at  $72^{\circ}\text{C}$  for 30 s, and a final melting curve. Given that skeletal muscle denervation differentially affects the expression levels of house-keeping genes (Nakao et al., 2015), the expression of the gene of interest in the denervation experiments was normalized using the geometric mean of multiple reference genes [glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA and  $\beta$ -actin] as previously recommended (Vandesompele et al., 2002). Otherwise, 18S ribosomal RNA was used as housekeeping gene for the *in vitro* experiments.

**In vitro stability assays.** Proteins extraction and mRNA degradation assays were performed as described previously (Chakkalakal et al., 2008). Briefly, proteins were extracted from 3 d denervated or control TA muscles using 500  $\mu\text{l}$  of a homogenization buffer (10 mmol  $\cdot$  L $^{-1}$  Tris, pH 8.0, 10 mmol  $\cdot$  L $^{-1}$  KCl, 1.5 mmol  $\cdot$  L $^{-1}$  MgCl $_2$ , 2.5% IGEPAL CA-630) containing protease inhibitor complete mini-tablets as per the manufacturer's recommendations (Roche Diagnostics). Protein extracts were centrifuged for 10 min at 3500  $\times$  g. After discarding the supernatant, pellets were subsequently vortexed and incubated on ice for 20 min in 100  $\mu\text{l}$  extraction buffer (20 mmol  $\cdot$  L $^{-1}$  Tris, pH 8.0, 450 mmol  $\cdot$  L $^{-1}$  NaCl, 10 mmol  $\cdot$  L $^{-1}$  EDTA, and protease inhibitor complete mini-tablets). After incubation, the pelleted fractions were centrifuged at 14,000  $\times$  g for 10 min and the supernatants (enriched in nuclear and cytoskeletal fractions) were collected and used for *in vitro* stability assays.

The RNA used in these assays was isolated from 3-d-old differentiated C2C12 myotubes using TriPure reagent (Roche Diagnostics). Degradation assays were performed by adding 0.2  $\mu\text{g} \cdot \mu\text{l}^{-1}$  of total RNA from C2C12 cells to 0.25  $\mu\text{g} \cdot \mu\text{l}^{-1}$  of TA muscle protein extracts and by incubating these two components into a degradation buffer (10 mmol  $\cdot$  L $^{-1}$  Tris pH 7.4, 10 mmol  $\cdot$  L $^{-1}$  KOAc, 2 mmol  $\cdot$  L $^{-1}$  MgOAc, 2 mmol  $\cdot$  L $^{-1}$  DTT, 0.1 mmol  $\cdot$  L $^{-1}$  Spermine, 1 mmol  $\cdot$  L $^{-1}$  ATP, 0.4 mmol  $\cdot$  L $^{-1}$  GTP, 10 mmol  $\cdot$  L $^{-1}$  Phosphocreatine, 40 U/ml creatine phosphokinase,

80 U SUPERNasin) for 20 min at 37°C. Time 0 was taken as RNA incubated in the degradation buffer without protein extracts. The reactions were stopped at different time intervals by addition of 250  $\mu$ l of Trizol. The RNA was then precipitated with isopropanol in the presence of 1  $\mu$ l of glycogen RNA grade (R0551, Thermo Fischer Scientific) as a carrier. The amount of AChR  $\beta$ -subunit and 18S transcripts remaining at each time point was determined by RT-qPCR analysis. Values were then plotted on a semi-logarithmic scale as a function of time. Four separate experiments were conducted, using four different TA muscle extracts. Half-life values were then determined relative to appropriate controls for each individual experiment. Relative half-life values were averaged and compared between samples.

**Plasmid preparation and electrotransfer of plasmid DNA.** *Escherichia coli* strain DH5 $\alpha$  was transformed with the plasmids 3'-untranslated region (UTR)-AChR  $\beta$ -subunit WT or 3'UTR-AChR  $\beta$ -subunit muted-AU-rich element (ARE) into pmirGLO luciferase vector using the heat shock method. Point mutations were performed with the QuikChange mutagenesis kit (Stratagene), according to the manufacturer instructions. The resulting mutations were confirmed by sequencing. Plasmid DNA was prepared using the Qiagen Mega-prep kit guidelines. Briefly, transformed bacteria colonies were selected for ampicillin resistance on LB growth plates. After selection and inoculation of a single colony in LB, the culture was grown for 12–16 h at 37°C under vigorous shaking. After assessment of their purity in a 1% agarose gel electrophoresis, the bacterial cells were harvested by centrifugation and the pellet was resuspended in the appropriate buffers. The suspension was then placed on a column and allowed to drain by gravity flow. After several washes, the plasmid DNA was eluted and precipitated with isopropanol. Mixed gently, the precipitated plasmid DNA was immediately centrifuged and the obtained pellet was washed with ethanol. Finally, the DNA pellet was air dried and redissolved in 500  $\mu$ l of 0.9% saline solution. Plasmid DNA concentration was measured using a spectrophotometer (Pharmacia GeneQuant) at 260 nm.

Under isoflurane anesthesia, TA muscles of mice were injected with 30  $\mu$ l of the solution containing either the 3'UTR-AChR  $\beta$ -subunit WT or the 3'UTR-AChR  $\beta$ -subunit mutated-ARE constructs. The injected solution contained a concentration of DNA equal at 1  $\mu$ g  $\cdot$   $\mu$ l<sup>-1</sup>. Thirty seconds after injection, six pulses (20 ms, 50 mA) were delivered to the muscles using an electroporator. In these experiments, a  $\beta$ -galactosidase cDNA plasmid (pCMV  $\beta$ -galactosidase containing the cDNA of the *E. coli*  $\beta$ -galactosidase gene driven by the cytomegalovirus CMV enhancer and early promoter elements) was coinjected and used to monitor transduction efficiency and to normalize the luciferase data. Mice were then allowed to recover for 7 d before denervation.

**Luciferase reporter assays.** The activity of luciferase was determined using the Luciferase Reporter Assay kit (Promega) according to the manufacturer's instructions. Briefly, 300  $\mu$ l of 1 $\times$  Passive Lysis Buffer was added to powdered TA muscles and homogenized at 4°C. Homogenates were subjected to three freeze–thaw cycles and centrifuged at 10,000  $\times$  g for 10 min at 4°C. Measurements were performed using a luminometer (Lumat LB 9507; Berthold Technologies).

**$\beta$ -Galactosidase activity assays.** The  $\beta$ -galactosidase activity was assayed using the  $\beta$ -Galactosidase Enzyme Assay System from Promega, according to the manufacturer's instructions. Briefly, 50  $\mu$ l of assay 2 $\times$  buffer (2 mmol  $\cdot$  L<sup>-1</sup> MgCl<sub>2</sub>, 100 mmol  $\cdot$  L<sup>-1</sup>  $\beta$ -mercaptoethanol, and 1.33 mg  $\cdot$  ml<sup>-1</sup> *o*-nitrophenyl- $\beta$ -D-galactopyranoside) were added to 20  $\mu$ l of homogenates on a 96-well plate. The plate was then incubated at 37°C for 1 h. The reaction was stopped by adding 150  $\mu$ l of 1 mol  $\cdot$  L<sup>-1</sup> sodium carbonate solution to each well. The absorbance of the samples was measured at 420 nm with a plate reader (Synergy, BioTek). Absorbance was plotted by linear regression against a  $\beta$ -galactosidase standard curve.

**Protein isolation and immunoblotting.** Proteins were extracted from powdered TA muscles by homogenization at 4°C in 1 $\times$  Passive Lysis Buffer (Promega). Homogenates were centrifuged at 12,000  $\times$  g for 20 min at 4°C, and the resulting supernatants collected and stored in aliquots at -80°C. Total protein content was measured using the bicinchoninic acid protein assay kit (Pierce BCA Protein Assay kit, Thermo Fischer Scientific) with bovine serum albumin as a standard. For Western

blotting, equal amounts of proteins (30  $\mu$ g) were loaded on 10% SDS-polyacrylamide gels. Proteins were transferred onto 0.2  $\mu$ m nitrocellulose membranes. Systematically, gel loading and transfer efficiency were ascertained by Ponceau S and Coomassie Blue staining, respectively. Membranes were then blocked with 5% skim milk in PBS containing also 0.05% Tween to avoid nonspecific binding, and they were incubated overnight at 4°C with primary antibodies. Blots were probed using antibodies (1:1000) directed against  $\beta$ -actin, HuR (Santa Cruz Biotechnology), phospho-p38 (Thr180/Tyr182), total p38 and phospho-MKK3(S189)/MKK6(S207; Cell Signaling Technology), total MKK3/6 (R&D Systems), MKP-1 (Millipore), MKP-5 and lamin A+C (Abcam), and  $\alpha$ -tubulin (Sigma-Aldrich). After washing with PBS containing 0.05% Tween, membranes were incubated with anti-mouse IgG light-chain-specific horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). After 3  $\times$  5 min washes with PBS with 0.05% Tween, chemiluminescent signals were revealed using ECL reagents (Pierce ECL Western Blotting Substrat, Thermo Fischer Scientific). The x-ray films (Thermo Fisher Scientific) were finally scanned and quantified with ImageJ analysis software (National Institutes of Health).  $\beta$ -Actin or  $\alpha$ -tubulin served to verify equal protein loading among samples.

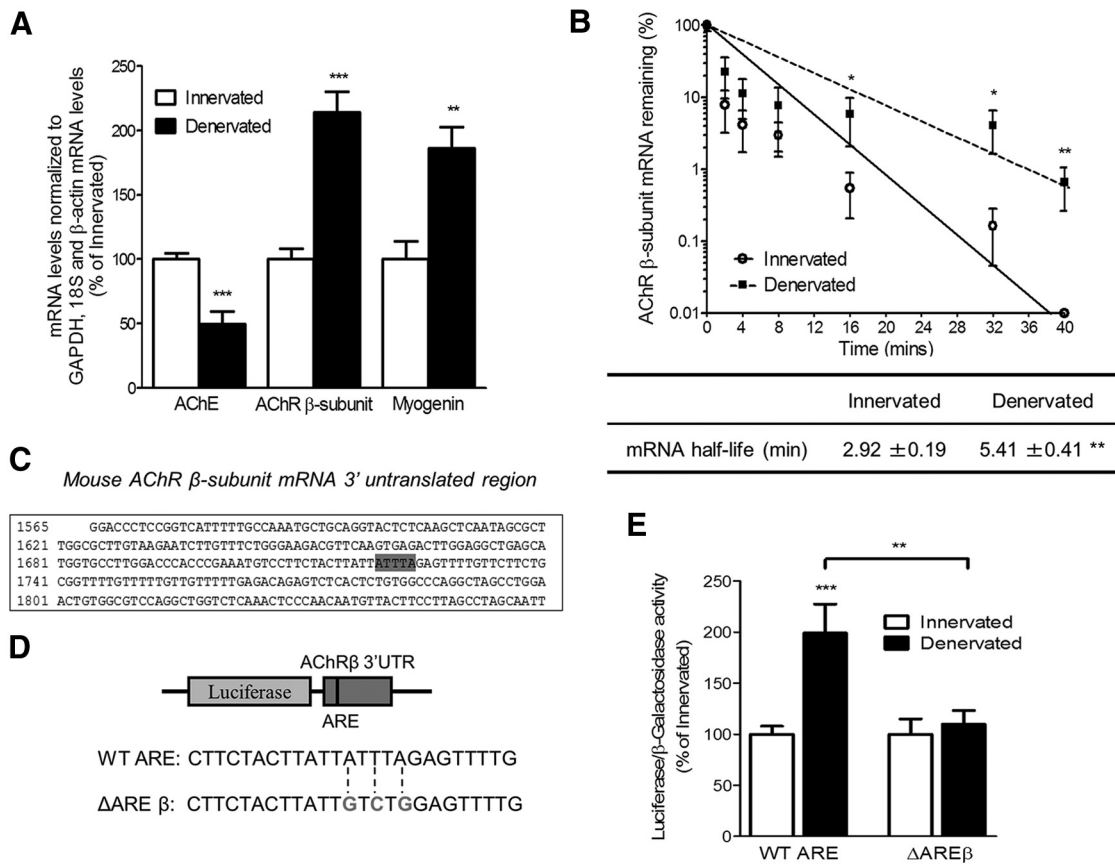
**Subcellular fractionation.** Control and 3 d denervated TA muscles were powdered in liquid nitrogen with a mortar and pestle. Subcellular fractionation was performed as previously described (Dimauro et al., 2012) with tubulin and lamin A+C used as cytoplasmic and nuclear fraction markers, respectively.

**Immunofluorescence.** TA muscle cross-sections (10  $\mu$ m) were cut using a microtome at -20°C and processed for immunofluorescence with the HuR (Santa Cruz Biotechnology) and laminin (Sigma-Aldrich) antibodies. Cross-sections were also stained with AlexaFluor 488-coupled  $\alpha$ -bungarotoxin (B13422, Life Technologies) to visualize AChRs. The MOM Immunodetection Kit (Vector Laboratories) was used to localize the primary antibodies. Slides containing tissue sections were mounted with Vectashield mounting medium (Vector Laboratories). Tissue sections were visualized using a Zeiss AxioImager.M2 fluorescent microscope and high-resolution images were acquired using ImageJ (NIH).

**RNA immunoprecipitation.** RNA immunoprecipitation (RNA-IP) was performed as recently described (Amirouche et al., 2013a). Briefly, cultured C2C12 myotubes were crosslinked with 1% formaldehyde in PBS for 10 min at room temperature. The reaction was stopped with a wash in ice-cold PBS. Equal amounts of whole-cell extracts were immunoprecipitated with Protein A agarose-bound rabbit anti-HuR antibody beads (Sigma-Aldrich) or rabbit IgG (Santa Cruz Biotechnology) as control. The beads were washed with modified RIPA buffer supplemented with protease and phosphatase inhibitors (Roche Diagnostics) and heated to 70°C for 1 h to reverse crosslinking. RNA was extracted from the immunoprecipitates using Tripure Reagent (Roche Diagnostics). Real-time RT-qPCR was performed as described above. RIP assays using TA innervated and denervated muscles were performed as previously described (Nechama et al., 2008).

**Cell culture, plasmids, and transfection.** Mouse C2C12 muscle cells (American Type Culture Collection) were plated in DMEM supplemented with 20% fetal bovine serum (MultiCell, Wisent), 1% penicillin-streptomycin (P/S), and 1% L-glutamine (Thermo Fischer Scientific) at 37°C and 5% CO<sub>2</sub> in air in 6-well culture dishes. At ~20–30% confluency, C2C12 myoblasts were transfected with si-HuR (kindly provided by Dr Imed Gallouzi, Department of Biochemistry, Goodman Cancer Center, McGill University, Montreal, QC, Canada) using jetPRIME (Polyplus transfection, VWR). This transfection was repeated 24 h following the first transfection when cells were at ~50–60% confluency. When cells reached 90–95% confluency, differentiation was induced for 3 d in a medium containing DMEM, 2% horse serum (PAA) and 1% P/S. To study the stability of the transcript, cells were treated with the transcriptional inhibitor actinomycin D (5  $\mu$ g  $\cdot$  ml<sup>-1</sup>; Invitrogen Life Technologies) and harvested at 2, 4, 6, or 8 h.

Myotubes were treated for 1 h with SB203580 (S8307, Sigma-Aldrich) or anisomycin (A9789, Sigma-Aldrich), both at the concentration of 10  $\mu$ mol  $\cdot$  L<sup>-1</sup>, to either inhibit or activate p38 MAPK, respectively. Control cells were incubated with dimethylsulfoxide (D5879, Sigma-Aldrich). In



**Figure 1.** Denervation increases AChR  $\beta$ -subunit mRNA stability via an ARE site. **A**, Endogenous mRNA levels of AChE, AChR  $\beta$ -subunit and myogenin following 3 d of denervation. GAPDH, 18S and  $\beta$ -actin were used as housekeeping genes (see Materials and Methods). Data are mean  $\pm$  SEM,  $n = 10$ –12 per group. \*\*\* $p < 0.001$  and \*\* $p < 0.01$ : significantly different from respective innervated group. **B**, *In vitro* AChR  $\beta$ -subunit mRNA stability assays. Quantification of the amounts of AChR  $\beta$ -subunit mRNA at each time point, is plotted in a semi-log scale as percentage of RNA seen at time 0. 18S was used as a control. \* $p < 0.05$ , and \*\* $p < 0.01$ : significantly different from the innervated group. **C**, Portion of the 3' UTR sequence of the mouse AChR  $\beta$ -subunit transcript with the ARE binding site (ATTTA) highlighted. **D**, Schematic representation of the construct containing the luciferase reporter followed by the AChR  $\beta$ -subunit 3' UTR sequence. The localization of the ARE motif of the AChR  $\beta$ -subunit is indicated. Below, the ARE motif sequence in WT 3' UTR (WT ARE) and the mutated nucleotides in the ARE motif ( $\Delta$ ARE $\beta$ ) are shown. **E**, Luciferase activity after denervation. Mouse TA muscles were injected and electroporated with the luciferase reporter construct containing either the WT AChR  $\beta$  3' UTR (WT ARE) or the mutated AChR  $\beta$  3' UTR ( $\Delta$ ARE $\beta$ ). After 7 d, TA muscles were denervated. Three days after denervation, TA muscles were excised and luciferase activity measured. To correct for variations in transduction efficiency, luciferase activity was normalized to  $\beta$ -galactosidase activity and the results are expressed as a ratio of luciferase/ $\beta$ -galactosidase. Data are mean  $\pm$  SEM,  $n = 10$ –12 per group. \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ : significantly different from WT ARE denervated and WT ARE innervated, respectively.

parallel experiments, myotubes were treated for 24 h with Calcitriol (71820, Cayman Chemical; at the concentrations of 1, 20, and 100 nmol  $\cdot$  L $^{-1}$ ). Control cells were incubated with ethanol. Then, cells were treated with actinomycin D (5  $\mu$ g  $\cdot$  ml $^{-1}$ ) for 2, 4, 6, or 8 h to inhibit transcription.

In separate experiments, C2C12 myoblasts at 50% confluency were transfected for 4 h with an expression plasmid containing MKK6-EE or pcDNA3.1 (kindly provided by Dr Alex MacKenzie, Apoptosis Research Center, Children's Hospital of Eastern Ontario Research Institute). At 90–95% confluency, C2C12 cells were differentiated into myotubes for 3 d, and then treated with actinomycin D (5  $\mu$ g  $\cdot$  ml $^{-1}$ ). Data are from 3/4 independent culture dishes.

**Statistical analysis.** All values are expressed as mean  $\pm$  SEM. Unpaired *t* tests, as well as one-way ANOVA with Fisher's *post hoc* tests were used to determine whether differences between the different group means were significant. Statistical analyses were performed using StatView v5.0 (SAS Institute). The  $\alpha$ -level of significance was set at 0.05 for all comparisons.

## Results

### Skeletal muscle denervation increases AChR $\beta$ -subunit mRNA stability via an ARE

To determine whether the stability of AChR  $\beta$ -subunit mRNAs is altered in denervated muscles, we used short-term denervation as an experimental model system. We first examined the levels of

several mRNAs 3 d postdenervation in mouse TA muscles. In this context, we show that denervation induces a  $\sim 2.1$ -fold increase ( $p < 0.001$ ) in the levels of AChR  $\beta$ -subunit mRNA (Fig. 1A). As control mRNAs for the effects of denervation, we also examined AChE and myogenin mRNAs. As expected, AChE mRNA levels decreased by  $\sim 50\%$  after denervation ( $p < 0.001$ ), whereas those encoding myogenin increased by  $\sim 86\%$  ( $p < 0.01$ ; Fig. 1A), consistent with previous studies (Eftimie et al., 1991; Witzemann and Sakmann, 1991; Maltin et al., 1993; Michel et al., 1994).

To test whether denervation causes a change in the stability of AChR  $\beta$ -subunit mRNAs, we performed *in vitro* stability assays (Chakkalal et al., 2008). The use of such an approach is warranted in this case as treatment of mice with actinomycin D (an approach to study changes in mRNA stability widely used with cultured cells, see below for example) is fraught with difficulties including premature death of the animals shortly (few hours) after actinomycin D injection as previously discussed (Ross, 1995). For the *in vitro* stability assays, total RNA from 3 d differentiated C2C12 myotubes was isolated and incubated with protein extracts obtained from innervated or 3 d denervated TA muscles for different time intervals in a degradation buffer. The amount of AChR  $\beta$ -subunit mRNAs remaining at each time

point was subsequently determined by RT-qPCR and used to calculate mRNA decay kinetics. In these assays, we observed that AChR  $\beta$ -subunit mRNAs decay at a slower rate in the presence of protein extracts from denervated TA muscle compared with incubation with protein extracts from innervated muscles (Fig. 1B). Quantitative assessment of half-life values revealed that AChR  $\beta$ -subunit mRNAs degraded at approximately twice the rate ( $p < 0.01$ ) in the presence of innervated muscle protein extracts indicating that denervation causes an increase in their stability (Fig. 1B). As a control, the analysis of 18S transcripts did not reveal any difference ( $p > 0.05$ ) in the rate of degradation upon incubation with innervated or denervated TA muscle protein extracts (data not shown). These results demonstrate therefore, that in addition to transcriptional events, an increase in mRNA stability also contributes to the abundance of AChR  $\beta$ -subunit transcripts in denervated muscles.

Because stabilizing sequences are often located in the 3'UTR of transcripts, we screened the sequence of the AChR  $\beta$ -subunit mRNA for known motifs involved in RNA stabilization/degradation. Specifically, the mouse AChR  $\beta$ -subunit transcript is 2151 nucleotides (nt) long, contains 1506 nt of coding sequence and a 3'UTR that is 531 nt long. Bioinformatics analysis using the online resource AREsite (Gruber et al., 2011) revealed that the  $\beta$ -subunit of the mouse AChR mRNA contains one ARE in its 3'UTR (Fig. 1C) making it an excellent candidate for controlling the stability of this transcript. In addition, sequence comparison across human, orangutan, rat, cow, and mouse revealed that this ARE found in the AChR  $\beta$ -subunit transcript, is conserved across multiple species.

To evaluate the contribution of the ARE in regulating the abundance of the AChR  $\beta$ -subunit mRNA upon denervation, we first generated a wild-type and an ARE-mutated form of the mouse full-length 3'UTR and inserted each one of these downstream of a luciferase reporter gene (3'UTR WT ARE and 3'UTR  $\Delta$ ARE $\beta$ , respectively) driven by a constitutively active promoter. The mutated form contains three point mutations in the ARE element (Fig. 1D). We then injected/electroporated mouse TA muscles with luciferase constructs containing either the 3'UTR WT ARE or the 3'UTR  $\Delta$ ARE $\beta$ , and denervated 7 d later. To monitor transduction/electroporation efficiency, luciferase activity was normalized to  $\beta$ -galactosidase activity. Our results, expressed as a ratio of luciferase to  $\beta$ -galactosidase activity, revealed a  $\sim 2$ -fold increase ( $p < 0.001$ ) in luciferase activity at 3 d post-denervation with the wild-type  $\beta$ -subunit 3'UTR construct (Fig. 1E). By contrast, mutations of the ARE prevented the denervation-mediated increase in luciferase activity ( $p < 0.01$ ) thereby highlighting the critical role of the ARE in mediating changes in AChR  $\beta$ -subunit mRNA expression in denervated muscle.

#### *Denervation increases expression of HuR and promotes its nuclear-cytoplasmic translocation*

Because AChR  $\beta$ -subunit mRNAs decay at a slower rate in denervated muscle and that the ARE serves as an essential element in regulating transcript levels, we sought to identify a RBP that could play a key role in such a process. In this context, it has been shown that the ubiquitously expressed protein HuR is important in differentiating, cultured muscle cells by interacting with ARE contained in the 3'UTR of transcripts, such as MyoD and myogenin (Figuroa et al., 2003; van der Giessen et al., 2003). Based on this, we therefore wondered whether denervation affects HuR expression.

Using RT-qPCR, we first observed an increase ( $\sim 44\%$ ) in HuR mRNA levels ( $p < 0.01$ ) following 3 d of denervation of

mouse TA muscles (Fig. 2A). To complement this analysis, we also measured by Western blotting, the levels of HuR protein. Our results show that HuR protein is also increased ( $\sim 38\%$ ;  $p < 0.05$ ) after denervation (Fig. 2A). During myogenesis, it is known that HuR is cleaved by caspase 3 leading to the production of a cleavage product (HuR-CP1) known to promote accumulation of HuR in the cytoplasm (Beauchamp et al., 2010). Furthermore, skeletal muscle denervation results in an increase in caspase-3 protein and enzymatic activity (Siu and Alway, 2005, 2006). Thus, we also examined whether denervation increases HuR-CP1 protein level. Western blotting experiments revealed that the levels of HuR-CP1 are significantly increased ( $p < 0.05$ ) 3 d after denervation (Fig. 2B).

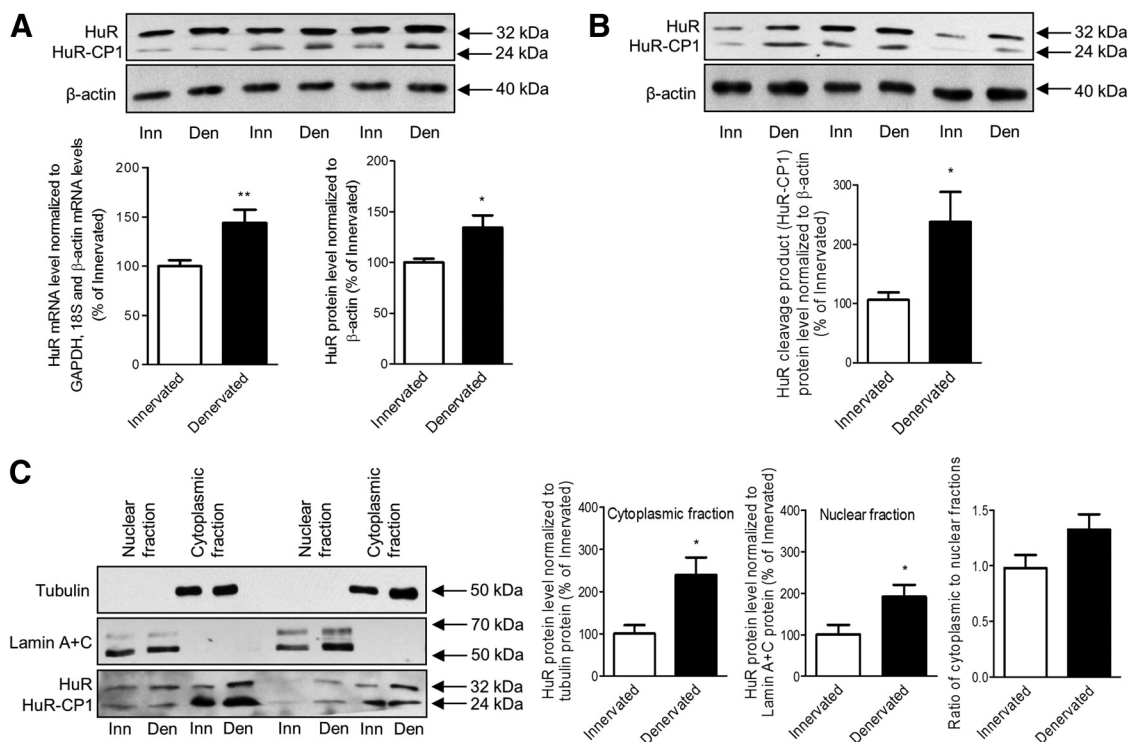
Increased cytoplasmic levels of HuR have been shown to correlate with its increased activity in ARE-mediated mRNA stability (Wang et al., 2000). To determine whether HuR protein is redistributed to the cytoplasm after denervation to potentially increase the stability of AChR  $\beta$ -subunit mRNAs, we conducted subcellular fractionation experiments and Western blotting on both nuclear and cytoplasmic fractions. The relative purity of the nuclear and cytoplasmic fractions was first evaluated by monitoring the presence of lamin A + C (expressed in nuclei) and tubulin (found in the cytoplasm; Fig. 2C). In these experiments, we noted that HuR protein levels were increased in both nuclear and cytoplasmic fractions after denervation. In fact, we observed a  $\sim 2.4$ -fold ( $p < 0.05$ ) and a  $\sim 2$ -fold ( $p < 0.05$ ) increase in HuR in the cytoplasmic and nuclear fractions, respectively (Fig. 2C). Although there is a global increase in the expression of HuR, there appears to be a greater increase in the cytoplasmic fraction as shown by analysis of the cytoplasmic/nuclear ratio in denervated versus innervated muscles (Fig. 2C), suggesting that HuR becomes enriched in the cytoplasm after denervation. Together, these results suggest that the stability of AChR  $\beta$ -subunit mRNAs is increased after denervation as a result of an increase in both HuR expression and in its cytoplasmic accumulation.

To complement these findings, we also performed immunofluorescence experiments using TA muscle cross-sections stained with antibodies against HuR. The data also revealed an accumulation of HuR in the cytoplasm of muscle fibers 3 d following denervation (Fig. 3A), reinforcing the notion that HuR translocates to the cytoplasm after denervation. Colabeling of AChR with  $\alpha$ -bungarotoxin confirmed an increase in the expression level of AChRs in extrasynaptic regions of denervated muscle fibers (Fig. 3B). Interestingly, we observed excellent extrasynaptic colocalization between AChR and HuR, especially in smaller perhaps more atrophied muscle fibers, indicating enhanced expression of AChRs in fibers with high levels of HuR.

#### *HuR interacts with AChR $\beta$ -subunit mRNAs*

To determine whether HuR controls the stability of AChR  $\beta$ -subunit mRNAs, we first examined whether HuR binds to AChR  $\beta$ -subunit transcripts in cultured muscle cells. To test this potential HuR-AChR  $\beta$ -subunit mRNA interaction, we performed RNA-IP experiments with myotubes. After formaldehyde crosslinking and HuR or IgG (used as a negative control) immunoprecipitation, coimmunoprecipitated RNAs were isolated and mRNA levels were determined by RT-qPCR. Our results show that in cultured differentiated muscle cells, HuR binds to endogenous AChR  $\beta$ -subunit mRNAs ( $p < 0.01$ ; Fig. 4A).

Based on these results, we also examined whether HuR binds to AChR  $\beta$ -subunit mRNAs *in vivo*. Given that the increased HuR expression after denervation is associated with an increased stability of AChR  $\beta$ -subunit mRNAs, we sought to also demonstrate



**Figure 2.** Denervation increases expression of HuR and induces its nuclear-cytoplasmic translocation. **A**, Endogenous mRNA levels of HuR (left) and representative immunoblot (top) and quantification (right) of HuR protein content 3 d postdenervation. GAPDH, 18S and  $\beta$ -actin were used as housekeeping genes for mRNA level (see Materials and Methods), whereas  $\beta$ -actin was used as a loading control for protein quantification. **B**, Representative immunoblot of HuR-CP1 protein content (top), and its quantification (bottom). Protein levels of HuR-CP1 were standardized using  $\beta$ -actin. **C**, Representative Western blots and their quantification for HuR protein expression from subcellular fractions of innervated (Inn) and denervated (Den) TA muscles. To verify the efficiency of the fractionation protocol, immunoblots were also probed for the cytoplasmic marker, tubulin, and the nuclear marker, lamin A+C. The same blots were used to determine HuR protein levels. Data are mean  $\pm$  SEM,  $n = 6-8$  per group. \* $p < 0.05$ , \*\* $p < 0.01$ : significantly different from respective innervation group.

that the interaction between HuR and AChR  $\beta$ -subunit mRNAs is increased in denervated muscle. To this end, we first cross-linked roughly chopped innervated and denervated muscles with formaldehyde. Equal amounts of whole muscle-cell extracts were then immunoprecipitated with Protein A agarose-bound anti-HuR antibody beads or IgG as control. Western blotting revealed the purity of the fraction after immunoprecipitation with the anti-HuR antibody (Fig. 4B, top). After crosslink reversal, the extent of interaction between HuR and AChR  $\beta$ -subunit mRNAs was analyzed by RT-qPCR. As shown in Fig. 4B, HuR does interact with AChR  $\beta$ -subunit mRNAs in innervated skeletal muscle ( $p < 0.01$ ). Importantly, the binding of HuR to AChR  $\beta$ -subunit transcripts is significantly higher ( $\sim 45\%$ ,  $p < 0.05$ ) in denervated versus innervated muscles.

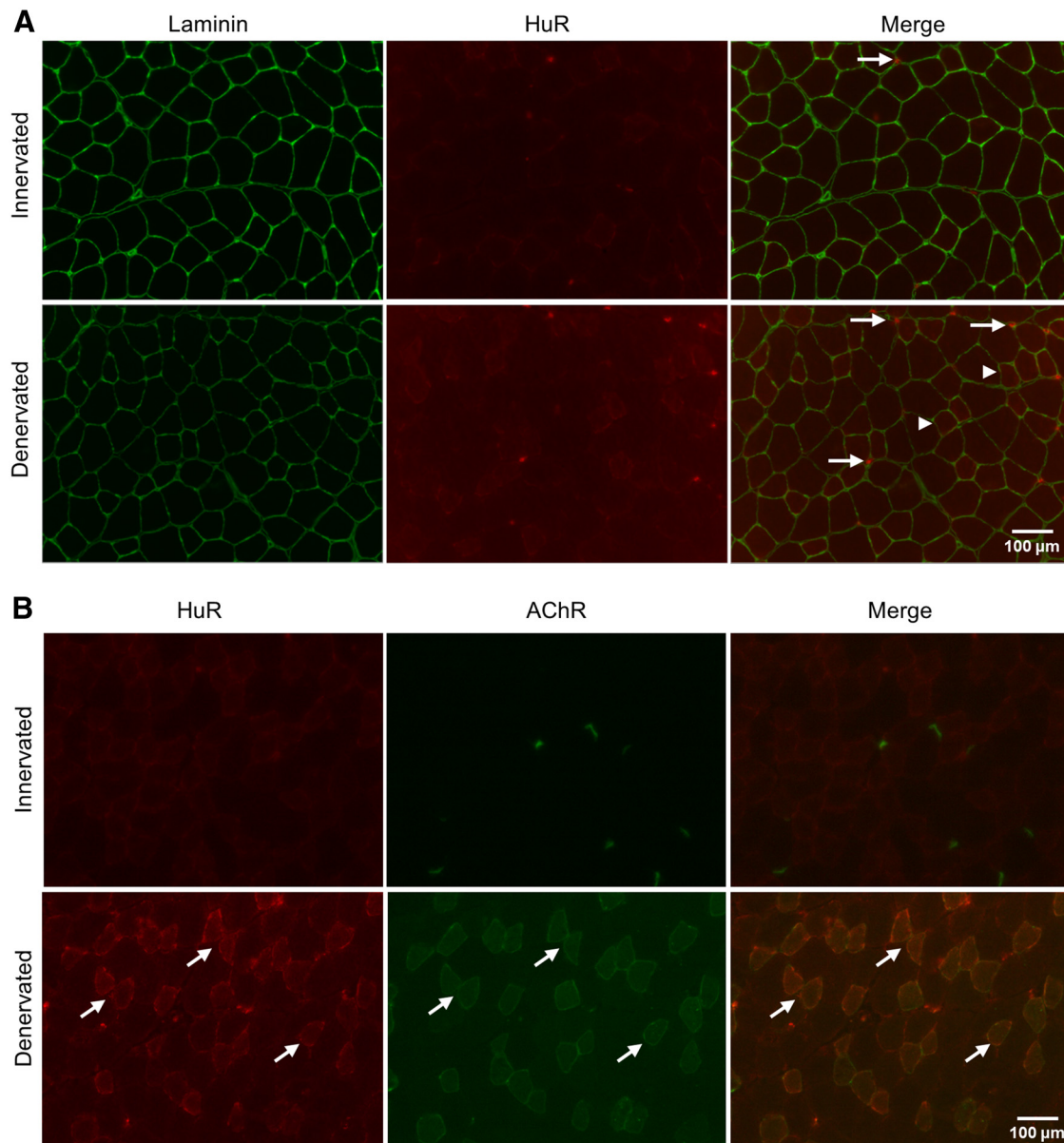
In separate experiments, we examined whether HuR functionally regulates levels of AChR  $\beta$ -subunit mRNAs and their stability. For this, we used a siRNA directed against HuR (si-HuR) and transfected C2C12 myoblasts with this construct. The same transfection was repeated 24 h later, and cells were then allowed to differentiate for 3 d. Silencing HuR was very efficient because it induced an  $\sim 80\%$  decrease ( $p < 0.001$ ) in HuR protein levels (Fig. 4C). In these cells, we noticed a significant ( $p < 0.001$ ) reduction in the levels of AChR  $\beta$ -subunit mRNAs (Fig. 4C).

To complement these experiments, we also determined the half-life of AChR  $\beta$ -subunit mRNAs in muscle cells transfected with the si-HuR construct. For this, we added the transcriptional inhibitor actinomycin D to cells after their transfection and differentiation. Then, the relative abundance of AChR  $\beta$ -subunit mRNAs was monitored at 0, 2, 4, 6, and 8 h. Quantitative deter-

mination of half-life values revealed that AChR  $\beta$ -subunit mRNAs degraded at  $\sim 2.3$  times the rate ( $p < 0.001$ ) in the presence of si-HuR (Fig. 4D). As a control, 18S transcript decay rates in the same cultured myotubes were not affected ( $p > 0.05$ ; data not shown). Altogether, these data indicate that by binding to the transcript, HuR positively regulates expression of AChR  $\beta$ -subunit mRNAs and their stability.

#### p38 MAPK signaling regulates AChR $\beta$ -subunit mRNA stability in vitro

An important question raised by the findings described above deals with the signaling events through which HuR regulates the stability of AChR  $\beta$ -subunit mRNAs. Previous work in motor neurons showed that anisomycin, an activator of p38 MAPK, causes a p38-dependent cytoplasmic accumulation of HuR (Farooq et al., 2009). Given this and our results implicating HuR in controlling the stability of AChR  $\beta$ -subunit mRNAs after denervation, we hypothesized that p38 MAPK activation in denervated muscles could increase the stability of AChR  $\beta$ -subunit mRNAs. To examine this, we first treated C2C12 myotubes with either an activator (anisomycin) or an inhibitor (SB203580) of p38 MAPK signaling. As shown in Fig. 5A, anisomycin ( $10 \mu\text{mol} \cdot \text{L}^{-1}$ ) increased ( $p < 0.001$ ) by  $>40\%$  the levels of AChR  $\beta$ -subunit mRNAs. By contrast, inhibition of p38 MAPK with SB203580 ( $10 \mu\text{mol} \cdot \text{L}^{-1}$ ) decreased ( $p < 0.001$ ) expression of AChR  $\beta$ -subunit transcripts (Fig. 5A). We also performed stability assays using actinomycin D and the activator or inhibitor of p38 MAPK. In this case, our results show that the stability of AChR  $\beta$ -subunit mRNAs is decreased ( $p < 0.01$ ) following p38 MAPK inhibition, whereas p38 MAPK activation increased ( $p < 0.1$ )



**Figure 3.** HuR colocalizes with AChRs in denervated muscle fibers. **A**, Representative TA muscle cross-sections stained with antibodies against HuR and laminin to depict the sarcolemma. Solid white arrows show HuR accumulation in nuclei and arrowheads represent HuR accumulation in cytoplasm. **B**, Representative TA muscle cross-sections stained with antibodies against HuR and labeled with  $\alpha$ -bungarotoxin to identify AChRs. Solid white arrows point to colocalization between HuR and AChR accumulation in extrasynaptic regions of denervated fibers.  $n = 4$  mice per group. Scale bar, 100  $\mu$ m.

transcript stability. Indeed, mRNA half-life measurements showed that AChR  $\beta$ -subunit mRNAs degraded at  $\sim 1.6$  time faster ( $p < 0.01$ ) after p38 MAPK inhibition while we observed a  $\sim 1.4$ -fold longer half-life ( $p < 0.05$ ) upon incubation of cells with anisomycin.

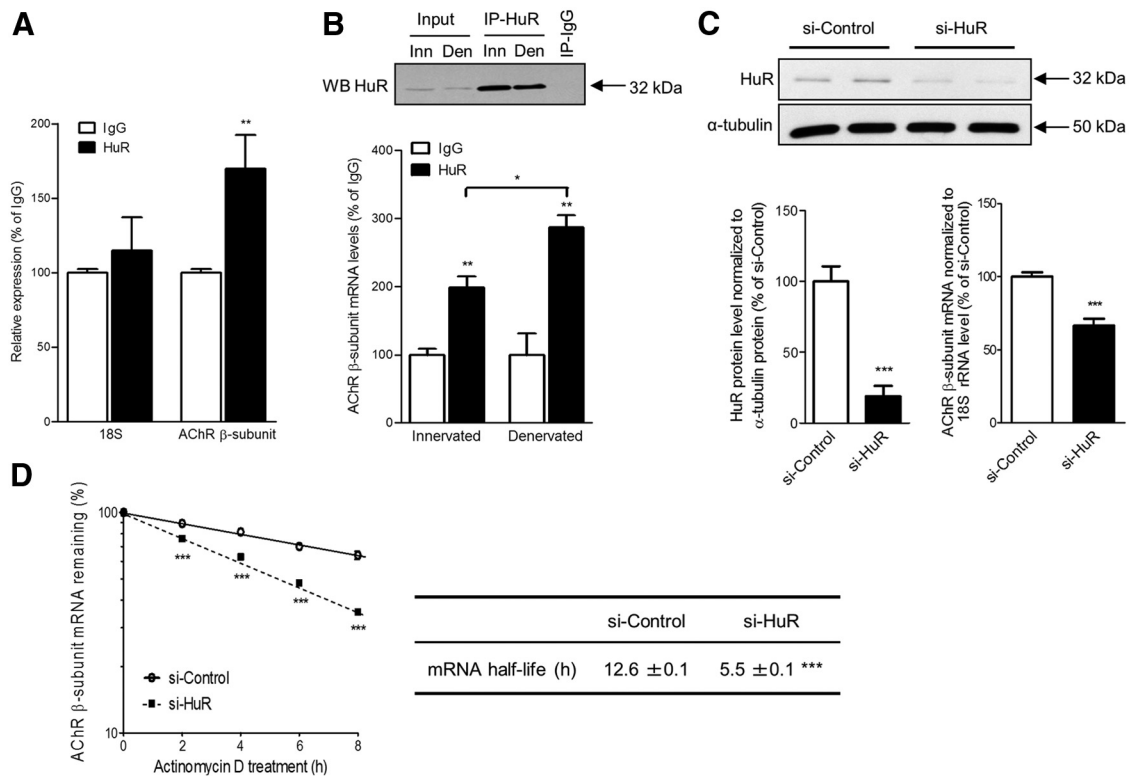
To confirm these results, we also treated myotubes with increasing doses of calcitriol (1, 20, and 100 nmol  $\cdot$  L $^{-1}$ ), another activator of p38 MAPK (Buitrago et al., 2006; 2013; Ronda et al., 2007), for 24 h. Our results show a dose-dependent increase in the phosphorylation status of p38 MAPK (Fig. 5B). Moreover, calcitriol increased ( $p < 0.05$ ) by  $\sim 20\%$  the endogenous levels of AChR  $\beta$ -subunit mRNAs. Finally, stability assays using actinomycin D showed an increase ( $\sim 1.9$ -fold,  $p < 0.001$ ) in the stability of AChR  $\beta$ -subunit mRNAs. Altogether, these findings obtained with a different p38 MAPK activator nam-

ely calcitriol, corroborate our data using anisomycin presented above.

#### *MKK3/6-p38 MAPK regulates AChR $\beta$ -subunit mRNA stability*

Based on these results, we wondered whether there was a change in p38 MAPK activation after denervation. Western blotting revealed that p38 MAPK is indeed activated ( $>50\%$ ,  $p < 0.01$ ) after denervation (Fig. 6A), leading us to further examine the role of p38 MAPK signaling in controlling the stability of AChR  $\beta$ -subunit mRNAs. First, and to uncover upstream signaling events involved in the activation of p38 MAPK induced by denervation, we examined expression of the kinases MKK3 and MKK6 which are known to phosphorylate p38 MAPK at threonine 180 and tyrosine 182. Interestingly, we saw after 3 d of denervation, an increase in the activity of MKK3 and MKK6 ( $p < 0.05$ ; Fig. 6B)





**Figure 4.** HuR binds and stabilizes endogenous AChR  $\beta$ -subunit transcripts in cultured myotubes and *in vivo*. **A**, RIP assays show that HuR interacts with endogenous AChR  $\beta$ -subunit mRNAs in cultured myotubes. 18S rRNA and IgG were used as controls. **B**, RIP experiments were performed using innervated (Inn) and denervated (Den) muscles. A representative Western blot confirming the quantity of immunoprecipitated HuR is shown (top, Input and IP). Data show the amount of AChR  $\beta$ -subunit mRNAs in each immunoprecipitate. Data are mean  $\pm$  SEM ( $n = 4$  per group). \* $p < 0.05$ , \*\* $p < 0.01$ : significantly different from innervated group and the corresponding IgG group, respectively. **C**, **D**, Stability of AChR  $\beta$ -subunit mRNAs in myotubes transfected with si-HuR. Representative immunoblot (**C**, left) and quantification (**C**, middle) of HuR protein content and endogenous mRNA levels of AChR  $\beta$ -subunit (**C**, right) after si-HuR transfection. 18S was used as a housekeeping gene for mRNA level and  $\alpha$ -tubulin as a loading control for protein quantification. After the myoblast transfection and differentiation into myotubes, cells were treated with actinomycin D ( $5 \mu\text{g} \cdot \text{ml}^{-1}$ ) for 2, 4, 6, and 8 h and AChR  $\beta$ -subunit mRNA half-life was measured (**D**). \*\*\* $p < 0.001$ : significantly different from si-Control group. Data are presented as percentage of control SEM ( $n = 3$ –5 independent experiments).

suggesting that such increases could sustain the increase in p38 MAPK phosphorylation seen after denervation.

To evaluate whether MKK6 increases the stability of AChR  $\beta$ -subunit mRNAs, we also transfected C2C12 cells with a MKK6-EE constitutively active mutant causing sustained activation of p38 MAPK signaling. Upon overexpression of MKK6-EE, we not only noted an increase ( $\sim 73\%$ ,  $p < 0.01$ ) in the phosphorylation of p38 MAPK as expected, but also we observed an increase in AChR  $\beta$ -subunit mRNA levels ( $\sim 46\%$ ,  $p < 0.05$ ; Fig. 6C). To determine whether constitutively active p38 MAPK regulates the stability of AChR  $\beta$ -subunit mRNAs, we also measured the degradation rate of these transcripts in transfected myotubes using actinomycin D. Under these conditions, we observed an increase ( $\sim 86\%$ ,  $p < 0.05$ ) in the stability of AChR  $\beta$ -subunit mRNAs in muscle cells transfected with the constitutively active MKK6-EE mutant.

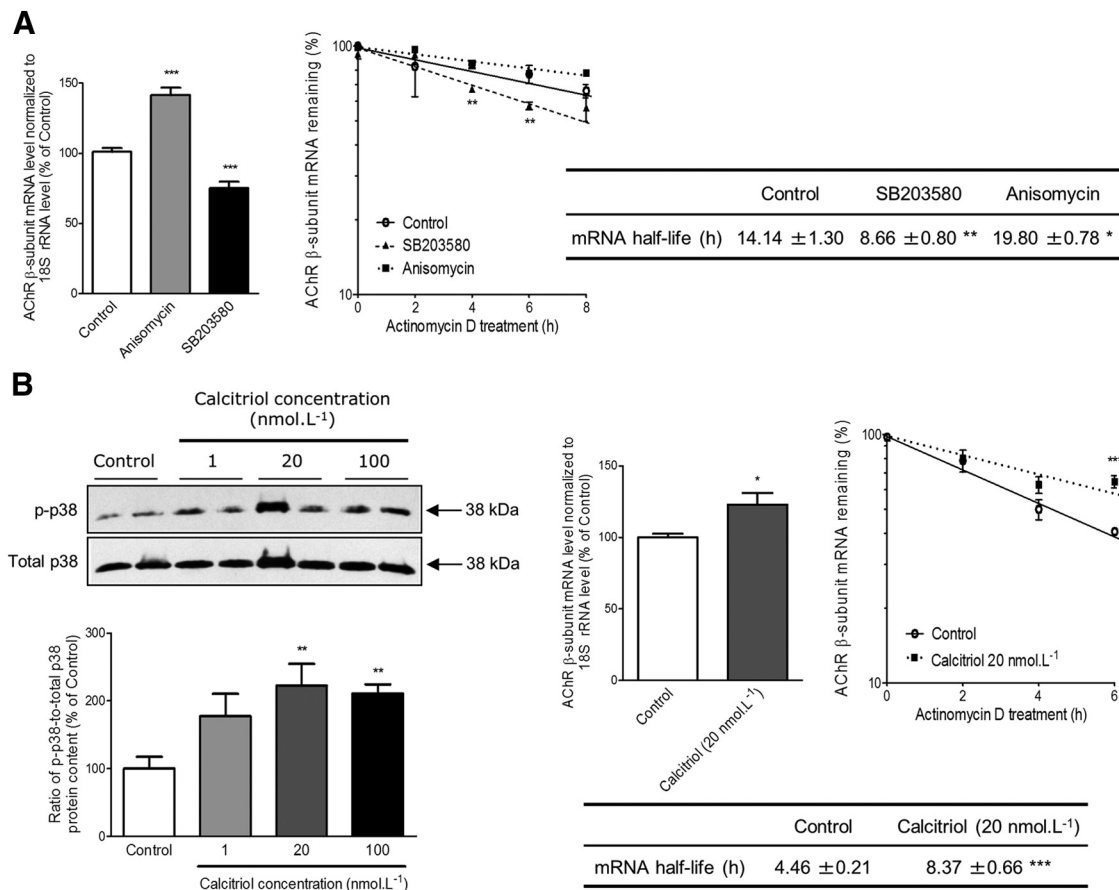
Finally, skeletal muscle is highly enriched in kinase phosphatases MKP-1 (Misra-Press et al., 1995) and MKP-5 (Theodosiou et al., 1999), known to be critical negative regulators of p38 MAPK signaling. Their contributions to the regulation of p38 MAPK after skeletal muscle denervation is currently unknown. In attempts to gain additional insights into upstream molecules engaged in p38 MAPK activation after denervation, we focused our attention on these two important kinase phosphatases. Analysis of denervated versus innervated muscles revealed a decrease ( $\sim 30\%$ ,  $p < 0.05$ ) in MKP-1 expression after denervation (Fig. 6D) with no changes in MKP-5 protein levels (Fig. 6E). Together,

these results suggest that the increased activation of p38 MAPK signaling induced by skeletal muscle denervation is at least partly due to the activation of the MKK3 and 6, and a concomitant reduction in the expression of MKP-1.

## Discussion

Transcriptional events have been described as the most important mechanism for regulating AChR mRNAs during myogenic differentiation and at the NMJ. Indeed, during muscle differentiation, AChR promoters are activated through E-boxes (Prody and Merlie, 1991, 1992; Jia et al., 1992; Simon et al., 1992; J. Tang et al., 1994; Bessis et al., 1995; Duclert and Changeux, 1995; Massari and Murre, 2000), which interact with myogenic factors to control transcription (Piette et al., 1990; Macpherson et al., 2006; H. Tang et al., 2006). At the mature NMJ, it was first suggested that neuregulin (NRG), which binds to ErbB receptors on the postsynaptic membrane, activates AChR gene transcription via the transcription factor GABP, which in turn binds to a regulatory element in AChR promoters termed the N-box (Schaeffer et al., 1998, 2001). However, mice lacking NRG/ErbB signaling express synaptic AChR genes (Escher et al., 2005), indicating that NRG signaling to muscle is not essential for AChR gene transcription. This, in turn, suggests the existence of parallel, yet elusive signaling pathways to control AChR gene transcription at the mature NMJ.

For years, denervation has been widely used as a model to study AChR gene regulation (Evans et al., 1987; Goldman et al.,

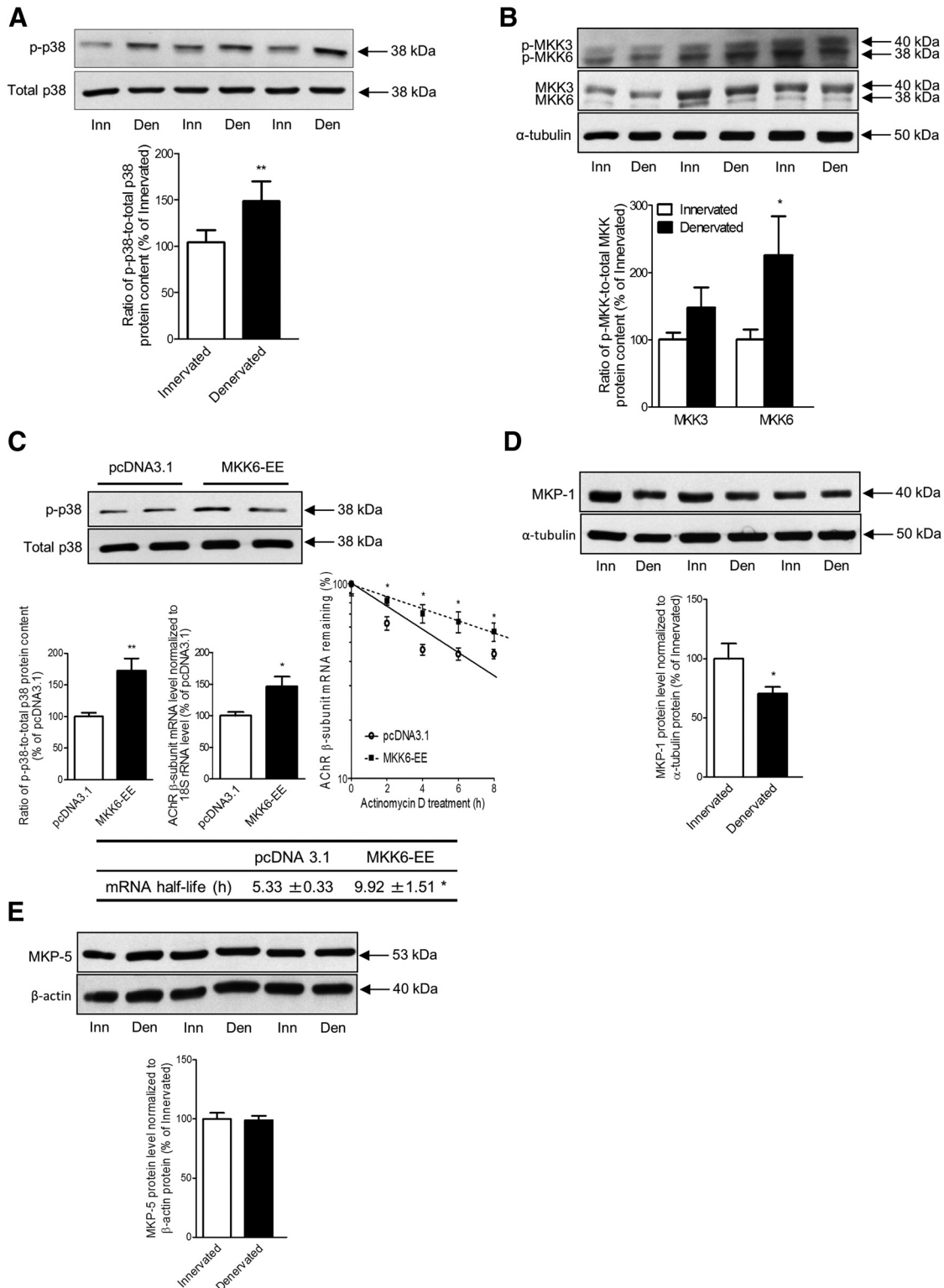


**Figure 5.** p38 MAPK signaling regulates AChR  $\beta$ -subunit mRNA stability *in vitro*. **A**, Levels of AChR  $\beta$ -subunit mRNA in 3 d differentiated myotubes treated with the activator of p38 MAPK, anisomycin, or its inhibitor, SB203580 (left) for 1 h. Stability of AChR  $\beta$ -subunit mRNAs in 3 d differentiated and drug-treated myotubes also treated with actinomycin D ( $5 \mu\text{g} \cdot \text{ml}^{-1}$ ) for 2, 4, 6, and 8 h (middle). AChR  $\beta$ -subunit mRNA half-life was measured and is shown on the right. **B**, Representative immunoblots of p38 phosphorylation (p-p38) and total p38 protein content (left), and quantification of the ratio p-p38/total p38 (bottom) after 24 h of calcitriol treatment (1, 20, and 100 nmol  $\cdot$  L<sup>-1</sup>) of 3 d differentiated myotubes. Levels of AChR  $\beta$ -subunit mRNA in 3 d differentiated myotubes treated for 24 h with calcitriol (20 nmol  $\cdot$  L<sup>-1</sup>). 18S was used as housekeeping gene. Stability of AChR  $\beta$ -subunit mRNAs in 3 d differentiated and drug-treated myotubes also treated with actinomycin D ( $5 \mu\text{g} \cdot \text{ml}^{-1}$ ) for 2, 4, and 6 h (right). AChR  $\beta$ -subunit mRNA half-life was measured and is shown in the table below. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ : significantly different from control group. Data are presented as percentage of control SEM ( $n = 3$ –4 independent experiments).

1988; Witzemann et al., 1989). Under these conditions, it has also been reported that transcriptional mechanisms regulate AChR gene expression (Schaeffer et al., 2001; Méjat et al., 2003; Kummer et al., 2006). Despite the abundant literature showing the importance of transcription in denervation-induced increases in AChR expression which occurs predominantly in extrasynaptic regions of muscle fibers (Hartzell and Fambrough, 1972; our study), such findings do not rule out that other mechanisms can also be involved. In fact, there is currently a lack of information on the role of post-transcriptional mechanisms in regulating synaptic mRNAs in muscle. Using multiple approaches, we show for the first time that denervation causes an increase in the stability of AChR  $\beta$ -subunit mRNAs. Moreover, we describe the critical role of the ARE located in the 3'UTR of AChR  $\beta$ -subunit mRNAs in controlling their stability. Such findings are in agreement with indirect evidence obtained by Tsay and Schmidt (1989) showing discordance between transcription and AChR mRNA abundance in denervated muscles (Duclert and Changeux, 1995), and with the notion that it takes more than transcription to regulate the levels and localization of synaptic mRNAs in muscle (Chakkalakal and Jasmin, 2003). Collectively, these data allow us to propose a model in which upon denervation, there is a burst of transcription of genes encoding AChR  $\beta$ -subunits that is accom-

panied by an increase in the stability of AChR  $\beta$ -subunit mRNAs as to maximize their expression.

Of relevance, our results show that the AChR  $\beta$ -subunit 3'UTR contains only one ARE which appears to play a critical role in mediating changes in AChR  $\beta$ -subunit mRNA expression. AREs are known to be key in mediating mRNA decay (Barreau et al., 2005; von Roretz et al., 2011b; Schoenberg and Maquat, 2012) and it has been estimated that  $\sim$ 8% of mRNAs contain AREs (Bakheet et al., 2006), which demonstrates their importance (Chen and Shyu, 1995; Matoulova et al., 2012). To mediate their effects on mRNA stability, AREs require interactions with specific RBPs. RBPs can either stabilize or destabilize target transcripts. For instance, AUF-1, TTP, TIA-1, and KSRP are known to typically destabilize mRNAs whereas the Hu family members HuB, HuC, HuD, and HuR, have marked effects in stabilizing transcripts (Apponi et al., 2011; von Roretz et al., 2011b). The reason that stimulated our interest in HuR is that this RBP is ubiquitously expressed including in muscle (Ma et al., 1996; Gallouzi et al., 2000; van der Giessen et al., 2003). Furthermore, previous work showed that HuR stabilizes MyoD, myogenin, and p21 mRNA levels (Figuroa et al., 2003; van der Giessen et al., 2003), and AChE transcripts in muscle cells (Deschênes-Furry et al., 2005).



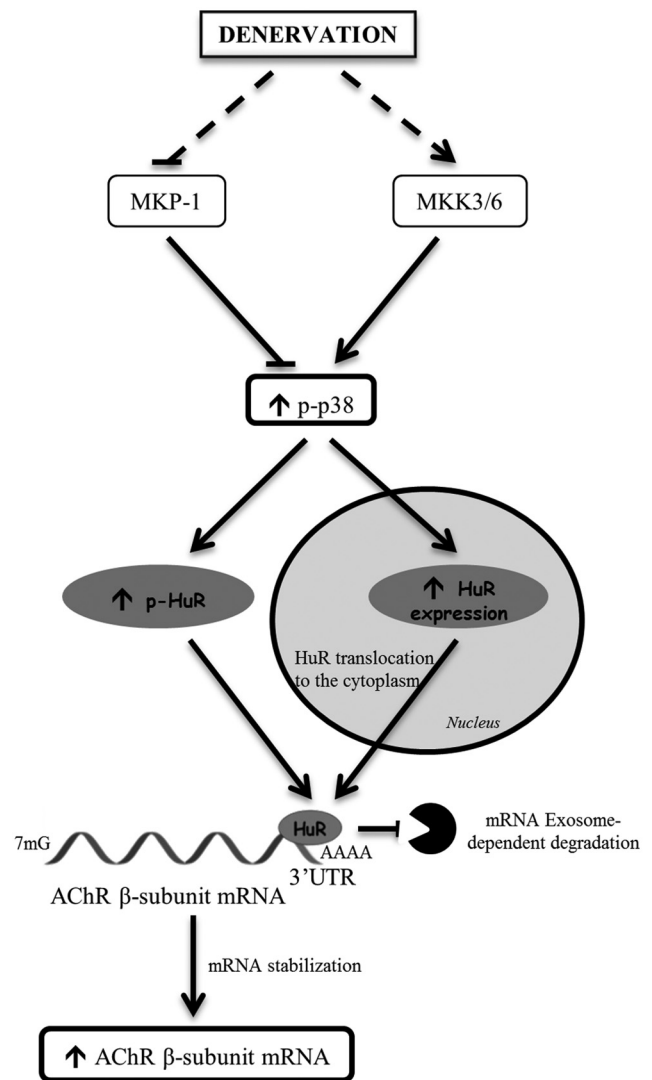
**Figure 6.** Denervation induces p38 MAPK signaling and regulates AChR  $\beta$ -subunit mRNA stability. **A**, Representative immunoblots of p38 phosphorylation (p-p38) and total p38 protein content (top), and quantification of the ratio p-p38/total p38 (bottom). \*\* $p < 0.01$ : significantly different from the innervated group. **B**, Representative immunoblots of MKK3/MKK6 phosphorylation (p-MKK3 and p-MKK6) and total MKK3/MKK6 protein content (top) and quantification of the ratio p-MKK/total MKK (bottom).  $\alpha$ -Tubulin was used as a loading control for protein quantification. \* $p < 0.05$ : significantly different from the innervated group. **C**, Stability of AChR  $\beta$ -subunit mRNAs in myotubes after transfection with the constitutively active mutant MKK6-EE. Representative immunoblots and quantification of p-p38 and total p38 protein content and levels of endogenous AChR  $\beta$ -subunit mRNAs (left). 18S was used as housekeeping gene. Transfected myotubes were treated with actinomycin D ( $5 \mu\text{g} \cdot \text{ml}^{-1}$ ) for 2, 4, 6, and 8 h (top, right) and AChR  $\beta$ -subunit mRNA half-life was measured and is shown in the table below. \* $p < 0.05$  and \*\* $p < 0.01$ : significantly different from pcDNA3.1 control group. **D**, Representative immunoblots of MKP-1 (top) and quantification of MKP-1 protein content (bottom). Protein levels of MKP-1 were standardized against  $\alpha$ -tubulin. The same membrane was used for MKP-1 and  $\alpha$ -tubulin. \* $p < 0.05$ : significantly different from the innervated group. **E**, Representative immunoblots of MKP-5 (top) and quantification of MKP-5 protein content (bottom). Protein levels of MKP-5 were standardized against  $\beta$ -actin. The same membrane was used for MKP-5 and  $\beta$ -actin. Values are mean  $\pm$  SEM ( $n = 4-8$  per group).

HuR belongs to the Elav-like protein family containing three conserved RNA recognition motifs (Venigalla and Turner, 2012). It is known that HuR shuttles between the nucleus and cytoplasm as myogenic differentiation proceeds (Fan et al., 1997; Peng et al., 1998; Figueroa et al., 2003; van der Giessen et al., 2003). By binding to ARE-containing mRNAs in the nucleus, HuR escorts transcripts through nuclear pores thereby providing protection during and after export to the cytoplasm (Fan and Steitz, 1998). During myogenesis, HuR is cleaved by caspases generating HuR-CP1 which binds to and blocks the HuR import factor transportin-2 (von Roretz et al., 2011a), thus facilitating accumulation of HuR in the cytoplasm (Beauchamp et al., 2010). Here, we observed an important increase in HuR following denervation. Additional experiments further revealed that denervation promotes the nuclear-cytoplasmic transport of HuR.

This novel post-transcriptional mechanism that we describe here, may be essential to ensure adequate expression of  $\beta$ -subunits and hence, of fully formed AChRs. In the absence of HuR, denervated fibers may not be able to synthesize sufficient AChRs to cause ACh supersensitivity, necessary for reinnervation, especially because AChR  $\beta$ -subunits may be rate-limiting in the assembly of AChRs (Claudio et al., 1989; Saedi et al., 1991). Similarly, one can also envisage that because HuR is important during myogenic differentiation (Figueroa et al., 2003; van der Giessen et al., 2003), its absence would impact synapse formation by limiting the ability of developing muscle fibers to express sufficient levels of AChRs. Given that the AChR  $\gamma$ - and  $\delta$ -subunit mRNAs also contain at least one ARE binding site, one could easily envisage a model of post-transcriptional coregulation of at least these AChR-subunits following denervation. Nevertheless, this coregulation via HuR would be limited to the  $\beta$ -,  $\gamma$ -, and  $\delta$ -subunits which raises the possibility that additional post-transcriptional mechanisms may be involved in controlling expression of the  $\alpha$ - and  $\epsilon$ - subunits.

During myogenic differentiation, phosphorylation of p38 MAPK increases (Galbiati et al., 1999; Zetser et al., 1999; Baeza-Raja and Munoz-Canoves, 2004), leading to transcriptional activation of MyoD and MEF2A and C (Ornatsky et al., 1999; Zetser et al., 1999). Furthermore, pharmacological activation of p38 MAPK in motoneurons causes a cytoplasmic accumulation of HuR (Farooq et al., 2009) with subsequent stabilization of target transcripts. We wondered whether p38 MAPK was activated after skeletal muscle denervation. As expected, we show increased phosphorylation of p38 MAPK postdenervation (Evertsson et al., 2014). In addition, we demonstrate that this activation of p38 MAPK signaling promotes changes in the stability of AChR  $\beta$ -subunit mRNAs. Such findings raise the question as to the nature of the signaling events involved in the increase of p38 MAPK in denervated muscle. We report for the first time that the p38 MAPK denervation-induced phosphorylation is associated with activation of MKK3/6 and with a concomitant decrease in MKP-1 expression. Previous work has shown that MKP-1 expression is also decreased in hindlimb muscle unloading (Misra-Press et al., 1995; Bey et al., 2003) and that muscle atrophy is associated with MKP-1 deficiency (Wu et al., 2006). Together, it appears therefore that denervation triggers an activation of upstream kinases while also inhibiting a known phosphatase, the cumulative impact being an activation of p38 MAPK signaling and redistribution of HuR.

Based on our results, we can depict a model of regulation in which the stability of AChR  $\beta$ -subunit mRNAs is increased by HuR following denervation via interaction with an ARE located in the 3'UTR. Denervation causes both an increase in the expres-



**Figure 7.** Model of post-transcriptional regulation of AChR  $\beta$ -subunit mRNAs following denervation. Skeletal muscle denervation increases the phosphorylation of p38 MAPK through activation of MKK3/6 and inhibition of MKP-1. p38 MAPK activation causes in turn an increased expression of HuR and its phosphorylation thereby promoting greater cytoplasmic localization of HuR. By interacting with the 3'UTR of AChR  $\beta$ -subunit mRNAs, HuR stabilizes the transcript which results in an increase in AChR  $\beta$ -subunit mRNA levels following denervation.

sion of HuR and in its nuclear-cytoplasmic export. Such translocation of HuR involves p38 MAPK whose activation levels are modulated following denervation by two pathways: an activation of upstream kinases and reduced expression of MKP-1 (Fig. 7). Remaining questions, therefore, concern the mechanisms by which denervation causes activation of MKK3/6 and inhibition of MKP-1, and whether such signaling changes play a role in other adaptive changes seen in denervated muscles. In this context, Fn14, the receptor of the tumor necrosis factor-related weak inducer of apoptosis (TWEAK), a muscle-wasting cytokine, is markedly increased after denervation (Mittal et al., 2010). Interestingly, TWEAK activates p38 MAPK in myotubes (Li et al., 2009) via MKK3/6, suggesting a link between denervation, TWEAK/Fn14, and MKK3/6/p38 MAPK. Given the well established role of calcium on AChR expression (Klarsfeld et al., 1989; Huang et al., 1994), one could also envisage a contribution of altered calcium dynamics as a trigger for activating p38 signaling in denervated muscle.

Our results do not preclude that the enhanced stability of AChR  $\beta$ -subunit mRNAs after denervation involves additional RBPs and/or micro-RNAs (Briata et al., 2005; Linker et al., 2005; Amirouche et al., 2013b). Therefore, the results presented here are only the beginning if the ultimate goal is to gain a full understanding of post-transcriptional events involved in controlling the stability of AChR  $\beta$ -subunit transcripts and other AChR subunit mRNAs. In this context, studying the expression and localization of RBP in skeletal muscle with a particular emphasis on the NMJ will not only yield novel findings related to events controlling the formation and maintenance of this synapse, but in addition, such studies could lead to the development of RNA-based therapeutics for neuromuscular disorders.

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