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Nicotinic stimulation induces Tristetraprolin over-production and attenuates inflammation in muscle

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ABSTRACT

Cholinergic signaling suppresses inflammation in blood and brain and attenuates apoptosis in other tissues, but whether it blocks inflammation in skeletal muscle under toxicant exposure, injuries and diseases remained unexplored. Here, we report nicotinic attenuation of inflammation and alteration of apoptotic protein expression pattern in murine muscle tissue and cultured myotubes, involving the RNA-binding protein, Tristetraprolin, and the anti-apoptotic protein, Mcl-1. In muscles and C2C12 myotubes, cholinergic excitation by exposure to nicotine or the organophosphorous pesticide, Paraoxon, induced Tristetraprolin overproduction while reducing pro-inflammatory transcripts such as IL-6, CXCL1 (KC) and CCL2 (MCP-1). Furthermore, nicotinic excitation under exposure to the bacterial endotoxin LPS attenuated over-expression of the CCL2 and suppressed the transcriptional activity of NF- B and AP-1. Tristetraprolin was essential for this anti-inflammatory effect of nicotine in basal conditions. However, its knockdown also impaired the pro-inflammatory response to LPS. Finally, in vivo administration of Paraoxon or recombinant Acetylcholinesterase, leading respectively to either gain or loss of cholinergic signaling, modified muscle expression of key mRNA processing factors and several of their apoptosis-related targets. Specifically, cholinergic imbalances enhanced the kinase activators of the Serine–Arginine splicing kinases, Clk1 and Clk3. Moreover, Paraoxon raised the levels of the anti-apoptotic protein, Mcl-1, through a previously unrecognized polyadenylation site selection mechanism, producing longer, less stable Mcl-1 mRNA transcripts. Together, our findings demonstrate that in addition to activating muscle function, acetylcholine regulates muscle inflammation and cell survival, and point to Tristetraprolin and the choice of Mcl-1 mRNA polyadenylation sites as potential key players in muscle reactions to insults.

1. Introduction

Muscle injuries and skeletal muscle diseases notably involve enhanced inflammation and apoptosis [1,2]. Acetylcholine (ACh) is the sole neurotransmitter in the neuromuscular junction (NMJ), where it binds to muscle nicotinic ACh receptors with the consequent depolarization of the muscle fiber, opening of voltage-gated Na^+ channels, and the triggering of an action potential in the fiber [3]. This process terminates when ACh is hydrolyzed by the enzyme Acetylcholinesterase (AChE). Therefore, AChE inhibitors such as organophosphorous nerve agents and pesticides enhance cholinergic signaling by potentiating the effect of a discrete quantity of ACh [4].

In other tissues, ACh was shown to regulate both inflammation [5,6] and cell death [7,8]. However, whether cholinergic signaling controls inflammation and apoptosis in skeletal muscles is yet unknown. Recent evidence suggests that skeletal muscles not only respond to immunological signals, but also operate as an active immunological tissue [9]. Muscle cells can act as antigen presenting cells [10] and express a rich repertoire of cytokines, chemokines (e.g. CCL2 (MCP-1) and CXCL1 (KC)) and their receptors [11]. For example, both type I and type II muscle fibers express IL-6 in response to muscle contractions, which then mediates exercise-induced inflammatory and metabolic effects [12]. Indeed, high levels of pro-inflammatory cytokines were observed in Duchenne muscular dystrophy [DMD, 13] and other myopathies such as dermatomyositis [2], but the underlying mechanisms remained unclear.

In peripheral macrophages, vagus-released ACh triggers an anti-inflammatory signal-transduction pathway. This effect is initiated by activation of the macrophage α7 nicotinic receptor (AChRα7) and culminating in suppression of NF- B-dependent transcription of pro-

Abbreviations: ACh, acetylcholine; AChE, Acetylcholinesterase; AChE-R, Acetylcholinesterase readthrough variant; AChRα7, α7 nicotinic receptor; ARE, AU-rich elements; DMD, Duchenne muscular dystrophy; miR, microRNA; NMJ, neuromuscular junction; PO, Paraoxon; RACE, rapid amplification of cDNA 3′ ends; shRNA, short hairpin RNA; TTP, tristetraprolin; UTR, untranslated region

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inflammatory genes. The suppression is achieved through inhibition of NF- B nuclear localization by phosphorylated I- B and activation of the JAK2-STAT3 pathway to disrupt the DNA binding activity of NF- B [14]. More recently this so-called cholinergic anti-inflammatory pathway [14] was demonstrated in a mouse model of DMD, where nicotine administration reduced skeletal muscle inflammation by suppressing the expression of cytokine production in infiltrating macrophages [15]. Yet, direct cholinergic suppression of inflammation in the muscle tissue itself was not yet addressed.

Cholinergic signaling often promotes survival. For example, in small cell lung carcinoma and pig retinal ganglion cells, cholinergic activation of either muscarinic [7] or nicotinic [16] receptors triggers phosphatidylinositol 3-kinase-mediated phosphorylation of AKT, resulting in the concomitant activation of proliferative transcription factors and inhibition of pro-apoptotic proteins. Additionally, in the developing rat hippocampus, cholinergic signaling promotes survival by enhancing expression of neurotrophic factors [17]. In muscles, inflammation is often accompanied by apoptosis, as was demonstrated in DMD mouse models and human patients ([e.g. in DMD mouse models and human patients, 18]), and in muscle injuries [1,19,20]. However, it remains unknown if cholinergic activation is also coupled to inflammation and apoptosis, and if so, what are the underlying mechanisms.

Many apoptosis-related genes undergo alternative splicing that yields variants with inverse functions (e.g. the prevalent, long anti-apoptotic Bcl-X and Mcl-1 variants and their rare, short pro-apoptotic variants [21]). Similarly, various genes in the pro-inflammatory pathway regulated by NF- B undergo alternative splicing, which may negatively regulate NF- B and reverse its effect [22]. Alternative mRNA processing is usually regulated by extra-cellular signals, and cholinergic signals are among them. For example, activation of brain muscarinic receptors by pilocarpine initiates alternative promoter choices in the BDNF gene and alternative splicing of many other transcripts (e.g. AChE, NMDAR1) [23,24]. Likewise, nicotinic stimulation in PC12 cells induces the expression of mRNA-binding proteins that bind tyrosine-hydroxylase and increases its stability [25], pointing at acquired changes in RNA processing as a putative mechanism downstream to cholinergic signaling.

A possible link between mRNA processing, inflammation and cell survival is the RNA binding protein Tristetraprolin (TTP). TTP is a zinc finger protein that binds AU-rich elements (ARE) in the 3′ untranslated region (UTR) of its target transcripts, and promotes their degradation by recruiting proteins involved in de-adenylation (e.g. xrn1) [26], de-capping (e.g. DCP2) [27], or microRNA (miR)-dependent degradation [28]. TTP-null mice develop a complex disorder that includes cachexia, myeloid hyperplasia, and joint and skin inflammation. Mice deficient in both TTP and TNF receptor 1 are protected from this disorder, suggesting that it is TNF-mediated [29]. Several other cytokine transcripts that contain AREs are regulated by TTP, such as IL-6 [30] and IL-2 [31]. Additionally, TTP regulates the mRNA levels of transcripts involved in cell proliferation and metastasis, for example in breast cancer processes and in human lung cancer cells [32,33]. TTP also binds the ARE of the apoptosis regulator, cIAP2 and promotes its degradation [34]. A recent article demonstrated that TTP mediates the cholinergic anti-inflammatory effect in macrophages [35].

Based on the central role of ACh in the NMJ on the one hand, and its involvement in prominent cellular processes implicated in muscular disease on the other hand, we set out to investigate cholinergic-mediated changes in mRNA processing in skeletal muscles, and challenge their importance in regulating cell death and inflammatory responses in muscle cells.

2. Materials and methods

2.1. Animal experiments

Groups of 4–6 male FVB/N mice were I.V. injected with 550 μg/kg Paraoxon (PO, 0.83× LD50, Sigma, St. Louis, MO, USA), 1000 U purified recombinant AChE-R [36] or a combination of both. Control mice were injected with an equivalent volume (100 μl) of sterile PBS. Where noted, mice were injected with 400 μg/kg nicotine (Sigma) or PBS. No additional supportive measures were provided. At the indicated time points, mice were sacrificed and solid tissue/whole blood was collected. Muscle tissues were immediately frozen on dry ice and subsequently stored at −80 °C until further use. Serum was prepared by centrifuging whole blood (15 min, 3000×*g*, 4 °C). Supernatants were removed and stored at −80 °C. All animal experiments were performed with the approval of the animal care and use committees at either Arizona State University or the Hebrew University of Jerusalem.

2.2. Cell culture

C2C12 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). They were grown in Dulbecco's modified Eagle's medium (+10% fetal calf serum), at 37 °C, 5% CO_2 in humidified chambers, and passaged every three to four days by trypsinization. To induce differentiation into myotubes, growth medium was replaced on Day 2, 4 and 6 with DMEM (+2% horse serum), and cells were used on Day 7 [37]. Differentiated cells were treated with 0.01–100 μM nicotine, 1.5 ng/ml–1.5 μg/ml LPS (Sigma), or a combination of both. For Mcl-1 protein quantification, cells were treated with 5 μM PO. Where indicated, Day 7 differentiated C2C12 myotube cultures were incubated in the presence of 50 μg/ml α-amanitin (Calbiochem, La-Jolla, CA) for the indicated duration.

2.2.1. Generation of shTTP cell line

To disrupt TTP expression we employed 'Block-it pol-II miR RNAi expression vectors' (Invitrogen, Carlsbad, CA, USA) specifically designed to target mouse TTP transcripts. These plasmids also included the gene for emerald GFP and blasticidin-resistance gene. A day after being sown in 6-well plates, undifferentiated C2C12 myoblasts were transfected with one of the three TTP or scrambled shRNAs (miR-like) encoding vectors, using lipofectamine-2000 reagent (Invitrogen). From Day 3 (when ~40% of the cells were observed to express emerald GFP) through Day 13, cells were grown in a medium containing 6 μg/ml blasticidin (Sigma) for selection. Following screening, we selected for further analysis the cell line that expressed the lowest levels of TTP mRNA and protein (shTTP). A cell line expressing high levels of the scrambled miR was similarly selected (shCTL). Whenever needed, frozen shTTP and shCTL cells were thawed, grown and differentiated in medium containing blasticidin. Unless stated otherwise, all cell culture materials were obtained from Biological Industries, Israel.

2.3. RNA extraction, quantitative RT-PCR and 3′RACE (rapid amplification of cDNA 3′ ends)

RNA was extracted using the RNeasy Minikit (Qiagen, Hilden, Germany) or the RNAqueous system with DNA-free treatment per manufacturer's instructions (Ambion, Austin, TX). Total RNA was reverse transcribed using random primers and Improm-II reverse transcriptase (Promega, Madison, WI, USA) or using the RetroScript system per manufacturer's instructions (Ambion).

Real-time quantitative RT-PCR (qRT) was performed on an ABI Prism 7900 Sequence Detector (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Each sample was tested 3 times. Deviant replicates were omitted if SD > 0.15 cycles. Data were normalized to 18S rRNA levels (muscle) or to a combined factor of Gapdh and α-tubulin levels. See Supplementary Table 1 for complete list of primers.

For 3′RACE, total RNA (400 ng) was similarly reverse transcribed using the 3′RACE-RT primer (Supplementary Table 1). cDNA fragments corresponding to Mcl-1 transcripts were PCR-amplified using *Ex-Taq* DNA polymerase (Takara, Shiga, Japan), the specific 3′RACE-Mcl-I and the universal 3′RACE-uni primers (Supplementary Table

1). PCR conditions were: 1x [95 °C – 3 min], and then 40x [95 °C – 45 s; 64 °C – 45 s; 72 °C – 2.5 min]. PCR products were analyzed using 1% agarose gel.

2.4. Cytokine and transcription factor ELISAs

Secreted IL-6 and CCL2 protein in conditioned medium from C2C12 cells (collected over a 24 h period) was assayed by ELISA using anti-murine IL6 and anti-murine CCL2 Abs, HRP-linked secondary Abs and 3,5,3′,5′-tetramethylbenzidine as a substrate according to the manufacturer's instruction (Ready-set-go! Kits, eBioscience, San-Diego, CA). Absorption (450 nm) was measured with SpectraMax 340PC plate reader (Molecular Devices, Sunnyvale, CA).

Activity of the transcription factor NF- B and AP-1 was assayed using the TransAM™ NFκB (p65) and TransAM™ AP-1 (c-fos) Transcription Factor ELISA kits (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.5. Immunoblotting

Total proteins from C2C12 cells that were lysed in SDS sample buffer were resolved by SDS-PAGE on 10% gels (BioRad, Hercules, CA) and blotted onto nitrocellulose membranes. Membranes were blocked with either 5% BSA or 5% skim milk, and then probed overnight at 4 °C with a rabbit polyclonal antiserum raised against the C-terminus of mouse TTP (SAK21B, 1:2500, a kind gift of Prof. Clark, Imperial college London, UK), rabbit polyclonal Mcl-1 Ab (1:800, Biolegend, San Diego, CA) or mouse monoclonal alpha-tubulin Ab (1:2000, Santa Cruz biotechnology, Santa Cruz, CA). Specifically-bound primary Abs were probed by incubation (1 h, room temperature) with HRP-conjugated secondary Abs (1:5000, Jackson Laboratories, West Grove, PA) and detected by enhanced chemiluminescence reagents (EZ-ECL, Biological industries, Israel). The TTP Ab detects two bands, probably reflecting different phosphorylation states [38,39]. TTP quantification included both bands together. Relative amounts of TTP and Mcl-1 proteins in each sample were determined by quantifying each band's densitometry and normalizing it to the relative amount of alpha-tubulin in the same sample.

2.6. Bioinformatics analysis of Mcl-1 3′-UTR

Polyadenylation sites in the mouse and human Mcl-1 genes were identified using the *PolyA_DB* database for mammalian mRNA polyadenylation [http://polya.umdnj.edu/PolyA_DB1/about.php, 40] and the Alternative Transcript Diversity database [http://www.ebi.ac.uk/atd/, 41].

2.7. Microarray experiments

Microarray design, RNA preparation and hybridization, scanning, basic analysis and normalization were performed according to [42,43]. Briefly, each microarray slide was hybridized with two distinct amplified, labeled muscle RNA samples, each composed from a pool of 4–6 mice. The resultant values are the relative ratio between hybridization signals, so data is always relative in nature rather than absolute, based on the "reference design" [44]. Supplementary Table 2 summarizes the different comparisons tested by the microarrays. To exclude dye-specific labeling differences, we performed dye-swapping tests [45]. Duplicate slides were used for each specific sample/label combination, so that each comparison includes four SpliceChip slides. Data filtration, normalization and analysis were performed with the Imagene software (Biodiscovery, CA, USA) and custom written Matlab programs.

2.7.1. Statistical analysis of microarray data

2.7.1.1. identifying significantly changed transcripts and groups of transcripts. Two approaches were used to identify transcripts and groups of transcripts whose expression levels were significantly altered [46]. Following the *discrete* approach, we first identified changed transcripts as those that were changed by more than 1.5-fold following treatment. Using the *continuous* approach, we searched for functional categories of transcripts that were differentially regulated as compared to all other transcripts on the SpliceChip. To this end, the cumulative distributions of the transcripts in each functional group were compared to those of the total population of transcripts on the chip, testing for significant differences between those two distributions by the two-sided Kolmogorov–Smirnov (KS) test [46].

2.7.1.2. Global correlations between treatments, time points and experimental systems. We calculated correlations between the changes in expression of all transcripts following the same treatment in different time points, and following different treatments at the same time points. Additionally, we compared the changes induced by AChE-R injection in muscles, to those induced by over-expression of the human AChE-R protein in murine P19 cells. These cells are embryonic carcinoma cells which upon induction with retinoic acid differentiate into neurons. We used those cells at differentiation on Day 5, at which they are defined as 'neuronal precursors' according to their morphology and gene expression [47]. All correlations were tested by Pearson's correlation test and represented by scatter plots and regression lines.

2.7.1.3. Correlations between mRNA processing factors and target genes. The expression changes of each apoptosis-related transcript in all 6 experimental comparisons (see Supplementary Table 2) were correlated using Pearson's correlation tests with parallel changes in each of the mRNA processing factors. 'target-factor' pairs that showed significant correlations ($p<0.0001$) were identified and presented in correlation matrices.

2.8. Other statistical analysis

Student's *t*-test was used for testing PO and nicotine effects on mRNA expression levels of Mcl-1, TTP, TNFα, IL-6, CCL2 and CXCL1 in muscles. 1-sample *t*-test was used for assessing TTP and IL-6 mRNA, and TTP protein expression in shTTP cells. Mann–Whitney test was used for quantification of TTP mRNA and protein, and Mcl-1 protein in differentiated C2C12 cells. A two-way ANOVA followed by Bonferroni post-hoc analysis was used for comparing CCL2 and CXCL1 mRNA and secreted proteins following treatments with different concentrations of nicotine and LPS in C2C12 cells, and for assessing nicotine effects on NF- B and AP-1 transcription activities in those cells.

All bars represents mean values, and error bars delineate standard error of the mean (S.E.M). Wherever bars represent fold change over control values, 1 stands for no change.

3. Results

Cholinergic stimulation can alter mRNA processing [24,25,48], improve cell viability [7,49], and regulate inflammation [5,6] in different tissues. Since muscle fibers are activated by ACh, we speculated that ACh regulates cell death and inflammation in this tissue as well, and that TTP can be a mediator of this regulation.

3.1. Nicotinic stimulation regulates pro-inflammatory transcripts in muscle cells

In cells of the immune system, cholinergic stimulation regulates inflammation through nicotinic receptors [5]. Thus, we set out to find if nicotinic stimulation can induce an anti-inflammatory response in muscles. We produced nicotinic stimulation in the muscle and tested whether it decreases the expression of pro-inflammatory cytokines. We observed pronounced reduction in the mRNA levels

of the pro-inflammatory chemokines, CCL2 (MCP-1) and CXCL1 (KC) 4 h after nicotine injection ($p<0.05$, Fig. 1A). These chemokine transcripts were already shown to be regulated by ACh signaling in non-muscle tissues [50,51], and are known targets of the mRNA binding protein TTP [30,52]. Similarly, IL-6 and TNF-α transcript levels were reduced 2 h after exposure to a sub-lethal dose of the AChE inhibitor, Paraoxon (PO), that also generates enhanced cholinergic transmission in the NMJ ($p<0.05$, Supplementary Fig. 1A).

To rule out contribution of non-muscle cells to the effects observed above, we turned to an in-vitro model of skeletal muscle cells. Congruent with the literature and our in-vivo experiments, CCL2 mRNA levels were induced by LPS exposure (reaching 11-fold increase over control 1 h after treatment), but this induction was attenuated by combined treatment with nicotine ($p<0.001$, Fig. 1B). Nicotine treatment alone reduced basal CCL2 mRNA levels within 1 h after treatment ($p<0.05$, Fig. 1B). In contrast, CXCL1 mRNA levels were only modestly induced by LPS (reaching 2-fold increase 4 h after treatment ($p<0.05$, Supplementary Fig. 2)) and the combined treatment with nicotine did not attenuate this affect. Interestingly, nicotine treatment alone induced, rather than reduced, CXCL1 expression 1 and 4 h after treatment ($p<0.001$). At the protein level, basal CCL2 secretion was suppressed in a dose-dependent and time-dependent manner by nicotine. At 48 h post-treatment, 0.01 μM nicotine reduced secreted CCL2 by 20.6% ($p<0.05$), reaching 68.1% suppression at 100 μM nicotine ($p<0.001$, Fig. 1C). Exposure to 1.5 μg/ml of the exogenous inflammatory inducer, LPS, induced 20-fold more prominent release of CCL2, which was likewise subjected to nicotine-mediated suppression ($p<0.001$, Fig. 1D). Similar, but less pronounced, nicotine-mediated suppression was seen for CXCL1 secretion, where nicotine at a concentration of 10 μM and more was required for suppression of basal secretion ($p<0.05$, Fig. 1E). 48 h after treatment, 100 μM nicotine reduced CXCL1 secretion by 30%. Interestingly, when cells were treated with LPS in a concentration required to enhance CXCL1 secretion (15 ng/ml), combined treatment with nicotine did not attenuate this induction (Fig. 1F). However, this LPS concentration is still much lower than the one used for demonstrating the attenuation of LPS-induced CCL2 secretion (1.5 μg/ml). Thus, further experiments are needed to determine whether nicotine can also suppress CXCL1 secretion after exposure to higher concentrations of LPS.

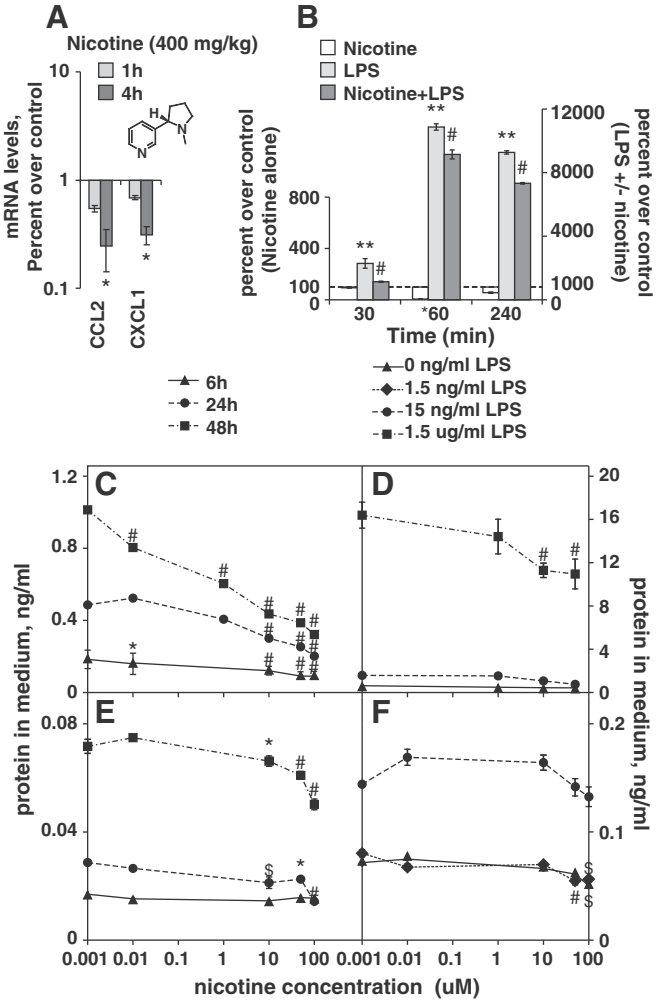

Fig. 1. The cholinergic anti-inflammatory effect in muscles. (A) Nicotine reduces CCL2 and CXCL1 mRNA expression in skeletal muscles, 4 h after treatment ($n=4$–6 per group, $^*p<0.05$, Student's *t*-test). (B) Nicotine (50 μM) attenuates LPS-induced expression of CCL2 transcripts in C2C12 cells ($n=4$ per group, $^*p<0.05$, $^{**}p<0.001$ in comparison to no treatment, $\#p<0.001$ in comparison to 100 ng/ml LPS alone) please note the different scales for nicotine treatment alone comparing with LPS treatments. (C) In C2C12 cells, secreted CCL2 protein is accumulated over time, but reduced by nicotine in a dose-dependent manner ($n=4$ per group, $^*p<0.05$, $\#p<0.001$, in comparison to no nicotine) (D) LPS-induced CCL2 secretion is also suppressed by nicotine, 48 h after treatment ($n=4$ per group, $\#p<0.001$ in comparison to no nicotine) (E) Basal CXCL1 secretion in C2C12 cells is attenuated by nicotine ($n=4$ per group, $^*p<0.05$, $\$p<0.01$, $\#p<0.001$ in comparison to no nicotine). (F) Nicotine does not attenuate 15 ng/ml LPS-induced CXCL1 secretion, 48 h after treatment ($n=4$ per group, $\$p<0.01$, $\#p<0.001$ in comparison to no nicotine). All analyses in panels C–F were subjected to Bonferroni post-hoc correction after a two-way ANOVA.

3.2. Tristetraprolin mediates the attenuating effect of nicotine in muscle cells

Next, we tested whether TTP can be the mediator of this cholinergic anti-inflammatory response in muscle cells. In muscles of treated mice, both nicotine and PO rapidly induced TTP mRNA expression ($p<0.05$, Fig. 2A and Supplementary Fig. 1B). In C2C12 cells, nicotine induced TTP mRNA levels yet more prominently ($p<0.05$, Fig. 2B and Supplementary Fig. 3), suggesting that cholinergic effect on TTP transcription occurs in muscle cells themselves and not only in macrophages as was previously suggested [15,35]. Importantly, we cannot completely rule out the possibility that nicotine and PO induce TTP expression in a mechanism independent of cholinergic receptors. However, the similar effects of nicotine, which is a direct cholinergic agonist and PO, which indirectly activate cholinergic signaling by inhibiting AChE, strongly propose a direct cholinergic involvement. TTP transcription induction led to a less pronounced, yet significant, increase in TTP protein levels ($p<0.05$, Fig. 2C). As in other cell types, treating C2C12 cells for 4 h with 10 μM nicotine treatment suppressed the activation of NF- B, and 0.5 h of 1 μM nicotine treatment suppressed AP-1 ($p<0.05$, Fig. 2D and E).

TTP induction could hence be either an essential step in the cholinergic anti-inflammatory pathway in muscles, or a secondary outcome of the suppression of immunologically-relevant transcription factors such as NF-kB and AP-1. To distinguish between these possibilities, we stably transformed C2C12 cells with an shRNA agent directed against the murine TTP transcript (shTTP cells). The shTTP cells express considerably lower levels of both TTP mRNA and protein in comparison to control cells transformed with scrambled shRNA (shCTL, $p<0.05$, Supplementary Fig. 4). Correspondingly, shTTP cells express higher levels than control cells of the TTP target, IL-6, likely reflecting reduced TTP-mediated degradation of IL-6 transcripts ($p<0.005$, Supplementary Fig. 4).

Then, we examined the effect of nicotine treatment and LPS exposure on pro-inflammatory transcripts in shTTP cells. We chose one cytokine, IL-6, and one chemokine, CCL2, as representative transcripts. In basal conditions, nicotine significantly reduced IL-6 and CCL2 expression in control, but not in shTTP cells ($p<0.05$, Fig. 2F), implying that normal levels of TTP are essential for the immunosuppressive effect of nicotinic stimulation. Importantly, nicotine treatment did not significantly increase IL-6 and CCL2 expression in shTTP cells. To our surprise, we failed to induce CCL2 and IL-6 expression in shTTP cells exposed to LPS, unlike shCTL cells which presented a robust response

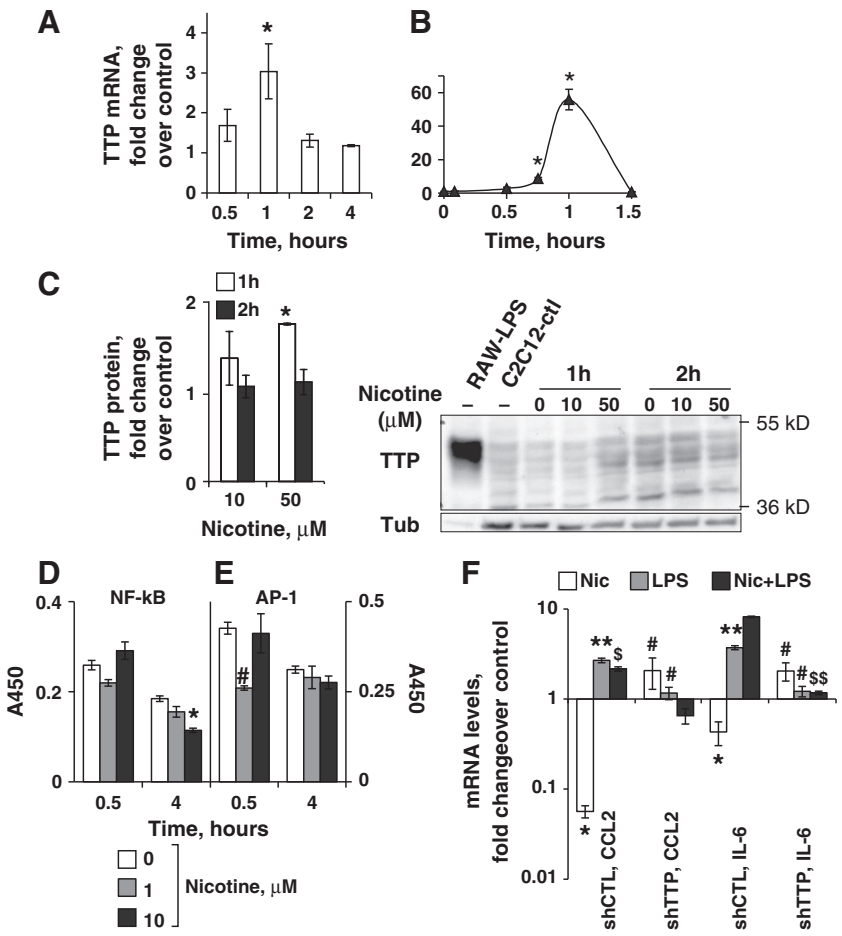

Fig. 2. TTP is essential for cholinergic regulation over muscle inflammation. (A) RNA was extracted from quadriceps muscle of mice treated with nicotine (400 μg/kg) in the indicated time points and resulting qRT-PCR demonstrated rapid induction of TTP mRNA (n = 4–6 per group, *p<0.05, Student's *t*-test). (B) Nicotine strongly induces TTP mRNA expression in C2C12 cells. Shown is a representative experiment, see a similar experiment in Supplementary Fig. 3. (n = 3 per group, *p<0.05, MW test). (C) Nicotine treatment also leads to TTP protein increase in C2C12 cells. LPS-treated macrophage cell line (RAW-LPS) served as positive control, TTP amounts were normalized to alpha-tubulin (tub) levels at the same samples (n = 3 per group, p<0.05, MW test, Tub = tubulin). (D, E) nicotine-treated C2C12 cells show reduced activity of NF- B and AP-1 compared to non-treated cells (n = 4 per group, *p<0.05; #p<0.001, Bonferroni post hoc analysis after a two-way ANOVA). (F) TTP knockdown impairs both the anti-inflammatory response to nicotine and pro-inflammatory response to LPS in C2C12 cells. C2C12 cells were stably transfected with either scrambled shRNA (shCTL) or TTP-targeting shRNA (shTTP). These cells were treated with nicotine (50 μM), LPS (500 ng/ml) or a combination of both. shCTL, but not shTTP cells showed reduction in CCL2 and IL-6 mRNA expression in response to nicotine treatment. Furthermore, shTTP cells did not increase CCL2 and IL-6 in response to LPS, and nicotine pre-treatment altered the response to LPS (attenuated CCL2, but potentiated IL-6 expression) in shCTL but not shTTP cells (n = 5–6 per group, *p<0.05, **p<0.001 in comparison to no treatment in the same cell line; #p<0.001 in comparison to shCTL cells; $p<0.05, $$p<0.001 in comparison to LPS treatment alone in the same cell line, Bonferroni post-hoc analysis after a two-way ANOVA).

(p<0.001, Fig. 2F). Furthermore, nicotine significantly altered LPS-induced CCL2 (p<0.05) and IL-6 (p<0.001) mRNA expression in shCTL, but not in shTTP cells. Interestingly, nicotine further enhanced IL-6 expression in shCTL cells, and not attenuated it. Thus, in addition to its above-demonstrated role in nicotine-mediated immunosuppression, TTP expression is important for cholinergic-independent, LPS-related cytokines expression.

3.3. Cholinergic signaling in the murine NMJ regulates the expression of mRNA processing factors and their apoptotic-related targets

Next, we aimed to test cholinergic regulation over muscle RNA processing and apoptosis, and the possible role of TTP in such processes. To simulate cholinergic changes in the NMJ, we injected FVB/N mice with either PO (550 μg/kg, 0.83× LD50), recombinant AChE-R (the usually rare, soluble AChE variant), which should reduce cholinergic signaling and elevate inflammation, or a combination of both. We extracted RNA from the quadriceps muscles two and six hours after treatment and used an homemade DNA microarray [42] to screen for differentially regulated mRNA processing factors, and their target genes that are involved in cell death and undergo alternative mRNA processing. We further quantified the expression of 13 transcripts represented on the array in all six experimental situations (treatment × time) by qRT-PCR and successfully validated 77% of the array results.

PO operates both as an anti-AChE and as a DNA alkylating agent. The consequent hyper-cholinergic signaling induces a feedback response of AChE over-production [53,54]. Nevertheless, when AChE-R is injected together with PO it serves as a scavenger for the PO molecules [36] and attenuates or even prevents some of the predicted PO-induced changes. Therefore, we surmised that AChE-R injection in muscles should induce a subset of those transcript modifications caused by PO, and that the difference between the effects of AChE-R and PO treatments should largely reflect those consequences of PO exposure which are non-cholinergic. Additionally, earlier work demonstrated that the motor effects of PO and/or AChE-R injections at the indicated doses are rapid and transient [36] and we expected the molecular changes to follow a similar kinetics. Correspondingly, injection of PO, recombinant AChE-R or a combination of both altered the expression levels of mRNA processing and apoptosis-related transcripts in comparison to saline injection. By two hours post-treatment, 11, 9.8 and 9% of the tested transcripts were changed by over 1.5-fold, and after 6 h, the percentages of changed transcripts dropped to 5.7,

5.3 and 3.3, respectively. Summation of changes across all transcripts in each experimental condition also indicated more changes two hours after treatment, with PO treatment alone causing more changes than the other treatments. (n = 4–6 per group, Fig. 3A). Thus, the timing of expression changes in mRNA processing factors and apoptosis-related transcripts correspond to the behavioral changes. Interestingly, not only that PO with or without AChE-R injection exerted more changes at the shorter time point, but also the total expression patterns of the two time points are negatively correlated ($p<0.001$ and $p=0.04$, respectively, Pearson's correlation test, Fig. 3B), indicating that 6 h after treatment, many of the transcripts show inverse changes in comparison to those seen 2 h after treatment. However, this pattern is not seen after

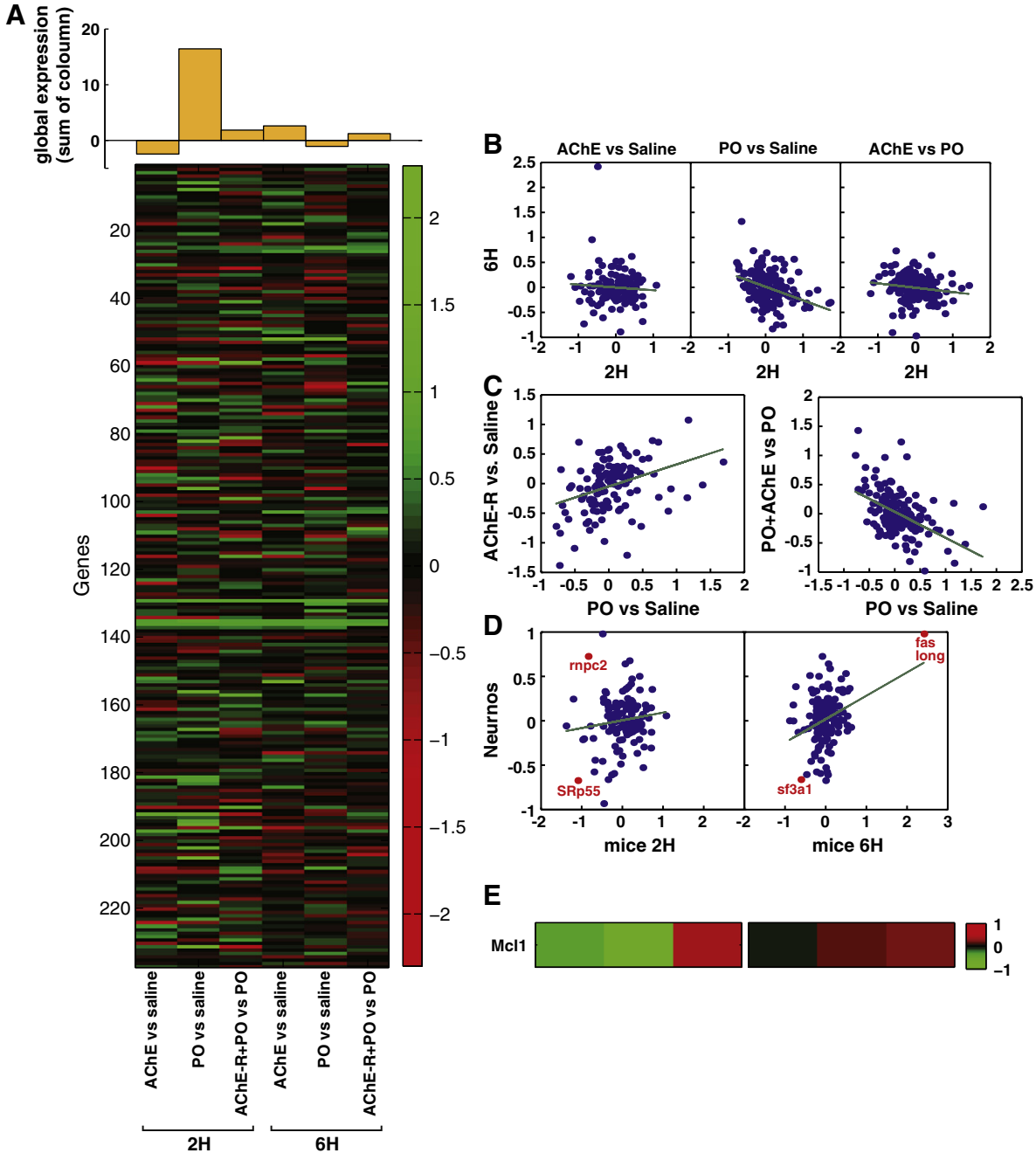

Fig. 3. Manipulating the cholinergic NMJ signaling alters muscle expression of mRNA processing factors. (A) Heat map of the 'SpliceChip' data. Red represents positive $\log_2$ of fold change (increases), green represents negative $\log_2$ of fold change (decreases) and black represents no change or missing data. Bar graphs represent the summary of $\log_2$ of fold changes across all transcripts in the array, for each experimental condition (n = 6 per group). (B) Rapid changes are later inverted or attenuated. Each point represents the $\log_2$ of the expression ratio between the indicated treatments for each transcript, 2 h (x-axis) and 6 h (y-axis) after injection. Regression lines and p-values (see Section 3.3) of Pearson's correlation test indicate significant negative correlations between time points for the changes induced by Paraoxon (PO) alone (middle), and for those exerted by combination of PO and AChE-R comparing to PO alone (right). However, changes induced by AChE-R alone were only attenuated, but not inverted, as indicated by the left graph. (C) AChE-R and PO injections exert similar changes, but combined injection inverts the PO effects. Shown are scatter plots representing the correlations between pairs of experiments, in the same time point (2 h). (D) Some transcripts were similarly affected by AChE-R excess in the NMJ and in neuronal precursor cultures. Each point represents the $\log_2$ of the expression ratio for each transcript, between AChE-R injection/transfection and control in mouse muscles (x-axis) and semi-differentiated P19 cells (y-axis), respectively. Transcripts that were changed in both systems by more than 1.5-fold are marked in red. Pearson's correlation test indicates significant correlation between changes induced by AChE-R transfections in neuronal precursors, and by AChE-R injections to the NMJ, 6 h after treatment. (E) Detailed heat map of changes in the expression of Mcl-1 transcript, as detected by 'SpliceChip'. The probe used for detection was later discovered as targeting the 'long' Mcl-1 variant. See Section 3.4 for details.

AChE-R injection alone ($p>0.05$). At two hours post injection, we noticed positive correlation between changes induced by AChE-R or PO ($p<0.001$, Pearson's correlation test, Fig. 3C), suggesting that AChE over-production caused part of the PO-induced changes. However, co-injected AChE-R inverted the PO-mediated effect on mRNA processing factors ($p<0.001$, Pearson's correlation test, Fig. 3C), in agreement with the expected effects on cholinergic transmission.

We also tested whether the cholinergic-mediated changes in the expression of mRNA processing factors were limited to specific subgroups of these factors, by comparing the changes in each functional group to the global changes in each experimental condition. Using the KS test, we could not identify any significantly changed category, indicating that the changes are transcript-specific and cannot be grouped according to specific functions in the mRNA processing machinery.

To test whether the cholinergic-mediated changes are specific to skeletal muscles, we compared the AChE-R-induced changes to those observed in neuronal precursor cell cultures (semi-differentiated P19 cells) transfected with the AChE-R expressing vector [42]. We noticed a significant correlation between the changes occurring six ($p<0.001$), but not two ($p>0.05$) hours after AChE-R injection to the NMJ to those observed in neuronal precursors 24 h after AChE-R transfection (Pearson's correlation test, Fig. 3D), indicating that to some extent, cholinergic stimulation exerts global changes in mRNA processing factors. Specifically, when setting the threshold for 'changed transcript' as 1.5-fold change, four transcripts were detected as 'changed' in both systems; the splicing factor, sf3a1, was reduced by excessive levels of AChE-R both in muscles and neuronal precursors, and the long variant of the pro-apoptotic transcript, Fas, was significantly induced in the two systems. Additionally, the SR protein, SRp55, was reduced two hours after AChE-R injection in the muscles and in the neuronal precursors, and the transcript encoding the RNA-binding protein, Rnpc2, was inversely affected in the two systems (Fig. 3D).

Next, we examined changes in mRNA processing factors across the experimental treatments and searched for corresponding changes in apoptosis-related transcripts known to undergo alternative mRNA processing that modifies their function, possibly reflecting functional relationships between the two. Out of 8100 valid 'mRNA processing factor-apoptotic target' pairs, 13 were significantly correlated with $p<0.0001$ (Supplementary Table 3). Within this subset of correlated pairs, we revealed complex relationships between cholinergic changes and apoptotic transcripts. For example, two hours after injection with either AChE-R or PO, the expression of the SR protein kinase, Clk1, was increased. Additionally, Clk1 was negatively correlated with the pro-apoptotic transcript Fas-long ($p=0.0086$), and positively correlated with the anti-apoptotic transcript, Bcl-X-com ($p=0.0048$), suggesting that enhanced cholinergic signaling induced a Clk1-mediated anti-apoptotic effect. However, both AChE-R and PO injections increased Clk3 expression, and this factor was positively correlated with caspase-6 ($p=0.01$) but negatively correlated with expression of the anti-apoptotic protein Mcl-1 ($p=0.0026$), suggesting a pro-apoptotic effect in this case. Validating this experimental investigation, we noticed a strong positive correlation between the expression of the alternative splicing factor, plrg1 [55], and the pro-apoptotic transcript bax-alpha ($p=0.005$). Earlier study already identified plrg1 in regulation of apoptosis, although it served as an anti-apoptotic regulator of p53-depdenent apoptosis [56] (Supplementary Fig. 5).

3.4. Cholinergic imbalances in the NMJ alter polyA site selection in the Mcl-1 gene

To obtain an in-depth view of one of the changes, we selected for further investigation the anti-apoptotic gene, Mcl-1, which prevents apoptosis by creating heterodimers with pro-apoptotic Bcl-2 proteins such as BAK and BAX, preventing their actions [57]. Both AChE-R and PO treatments caused suppression of Mcl-1, and the combined treatment inverted this effect (Fig. 3E). Primers targeting the extreme 3′ end of the transcript confirmed these results. Importantly, primers targeting a more 5′ part of the transcript did not show such changes, suggesting a 3′-specific effect ($p<0.001$, Fig. 4A). In-silica analysis of the murine Mcl-1 genomic region revealed three potential alternative polyadeylation sites which are conserved in humans and are assumed to yield three distinct transcripts that share an identical coding region but are different in the length of their 3′ UTR (Fig. 4B). These "short" (S), "intermediate" (I) and "long" (L) UTR-containing species are all present in mouse skeletal muscle, as demonstrated by 3′RACE (Fig. 4C). In conclusion, cholinergic changes modulated Mcl-1 polyadenylation site selection in skeletal muscles.

Since this is the first experimental demonstration of selective regulation over the 3′ region of Mcl-1, we explored possible differences between the variants. In differentiated C2C12 murine myotubes exposed to the RNA polymerase II inhibitor, α-amanitin, this revealed that Mcl-1 transcripts with the long-3′-UTR were more rapidly degraded compared with transcripts with short-3′-UTR (Fig. 4D). Also, western blotting of Mcl-1 usually yields two distinct bands, which result from either alternative splicing [58] or post-translational modification [59], and PO treatment elevated the expression of these two Mcl-1 forms ($p<0.05$, Fig. 4E). Together, these findings suggest that PO treatment reduces the selection of the less stable long variant, leading to increased selection of the other, more stable transcripts which results in enhanced accumulation of the anti-apoptotic Mcl-1 protein. Supporting this conclusion, the Mcl-1 3′ UTR includes no less than eight AUUUA RNA destabilizing sequences (ARE pentamers). Of these, one was in the common 3′-UTR, four in the region common to the long and intermediate variant, and the remaining three were in the unique long UTR. Thus, the long variant contains as many as eight ARE pentamers. Furthermore, one of the pentamers unique to the long variant is actually a part of the octamer UUAUUUAU, similar to the 'classic' UUAUUUAUU nonamer identified as a strong TTP binding motif (Fig. 4B). The presence of several pentamers and one octamer points at Mcl-1 long variant as a potential TTP target [60–62].

4. Discussion

We found that nicotinic stimulation suppressed the expression of pro-inflammatory cytokines in mouse muscle tissues and myotube cell culture. This suppression was accompanied by enhanced expression of the 3′ mRNA processing factor, TTP, and was abolished in cells with ablated TTP expression. High throughput screening revealed that cholinergic changes in the NMJ rapidly altered muscle transcript levels of several mRNA processing factors, with most of these changes being reversible within 6 h. Interestingly, some of the changes were correlated with alterations in apoptosis-related transcript levels. Specifically, injection of the AChE inhibitor, PO, modulated the 3′ mRNA processing pattern of the anti-apoptotic gene, Mcl-1, switching from selection of the most distant polyadenylation site and correspondingly suppressing the production of the long and less stable Mcl-1 mRNA variant, which carries eight ARE pentamers. These findings and the inverse correlation between TTP and the long Mcl-1 variant are compatible with the hypothesis that TTP mediates cholinergic control over inflammation and apoptosis in muscles (Fig. 5).

In macrophages, cholinergic stimulation regulates inflammation through a specific subtype of nicotinic receptors, namely AChRα7 [5]. Traditionally, it is believed that the NMJ of adult mammals express other subtypes of nicotinic receptors. However, AChRα7 expression was detected in chick and rat skeletal muscle during development and denervation [63]. Moreover, immunohistochemistry in human tissues revealed moderate AChRα7 staining in skeletal muscles (http://www.proteinatlas.org/ENSG00000175344/normal/skeletal+muscle). Additionally, it is possible that the nicotine and/or PO treatments by themselves alter the composition of nicotinic receptors in the NMJ. In any case, we cannot rule out that the inducing effects of nicotine and PO

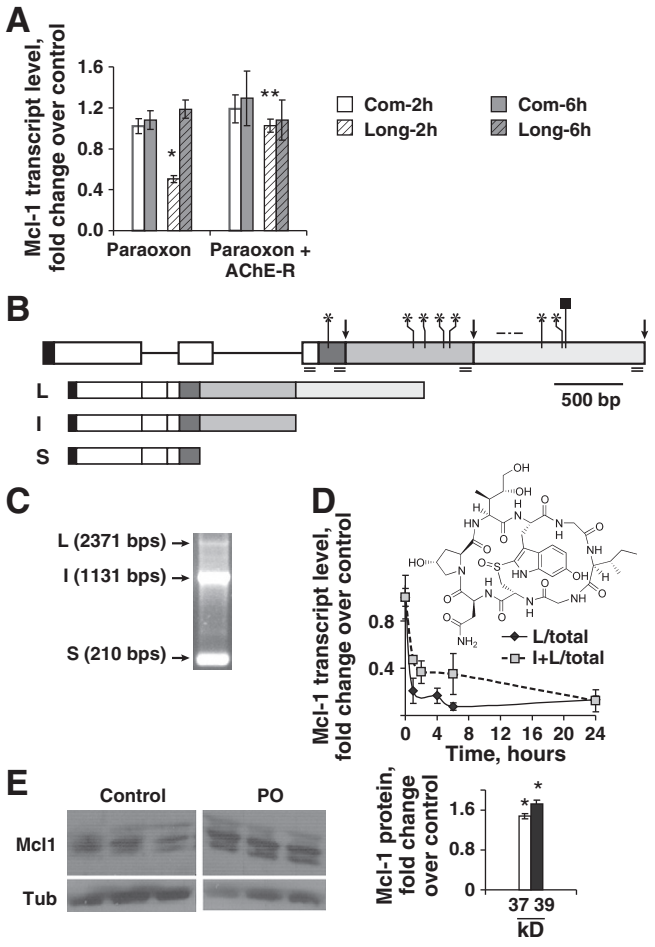

Fig. 4. Alternative selection of polyadenylation sites in the Mcl-1 gene. (A) qRT-PCR confirmed 'SpliceChip' results for the long variant of Mcl-1, and compared them with the total expression of Mcl-1 transcripts: PO exposure induces selective decreases in the mRNA expression of the "long" variant, but not in total Mcl-1 mRNA ('com'), 2 h after injection. This reduction is prevented by pre-injection of recombinant AChE-R (1000U), (n = 5–6 per group, *$p<0.001$ in comparison to control, **$p<0.001$ in comparison to PO treatment alone, Student's *t*-test). (B) The murine Mcl-1 gene has 3 conserved alternative PolyA sites in tandem: Coding regions are marked in white. 5′ UTRs are colored in black, 3′ UTRs are colored with gray gradient. Arrows indicate the three PolyA sites, and the resulting 3′-UTRs are colored with different shades of gray. Asterisks indicate the location of ARE pentamers, dark square indicates the UUAUUUAU octamer. The three possible transcripts are depicted below (L = long variant; I = intermediate; S = short). Upper dashed line shows the location of the probe used in the array. Double lines indicate the location of primers used for 3′RACE (See below). (C) The 3 Mcl-1 variants, resulting from cleavage at different PolyA sites, are evident in mouse muscles, as revealed by 3′RACE employed on RNA extracted from naïve mice (shown is a representative image, bps = base pairs). (D) The 'long' variant is less stable. Shown are mRNA levels of the long transcript (L) alone in comparison to the long and intermediate variants together (I + L), both normalized to total Mcl-1 mRNA, at different time points post-treatment with the RNA polymerase inhibitor, α-amanitin (The ratio between transcripts at time = 0 is defined as 1). (E) PO treatment elevates total Mcl-1 Protein levels in cells. Western blotting with anti-Mcl-1 antibody yields two bands (37 kD and 39 kD, see Section 3.4 for explanations). Quantification of each band shows similar induction by PO. Each sample was normalized to its own alpha-tubulin (tub) amount. The image shows 3 distinct biological replicates for each treatment group. All samples in the image were blotted on the same gel. The internal part of the gel was excised out of the image as it contains irrelevant samples (n = 3 per group, *$p<0.05$ in comparison to control, MW test).

on TTP expression are mediated by non-α7 nicotinic receptors in muscle cells, and the question of the identity of the specific nicotinic receptors involved in anti-inflammatory response in muscles is yet to be answered. Furthermore, the specific mechanism by which nicotinic receptors convey the TTP-inducing effect is still unclear.

Investigating the effect of nicotinic stimulation on pro-inflammatory cytokines involved treating C2C12 cells with nicotine and LPS. Nicotine

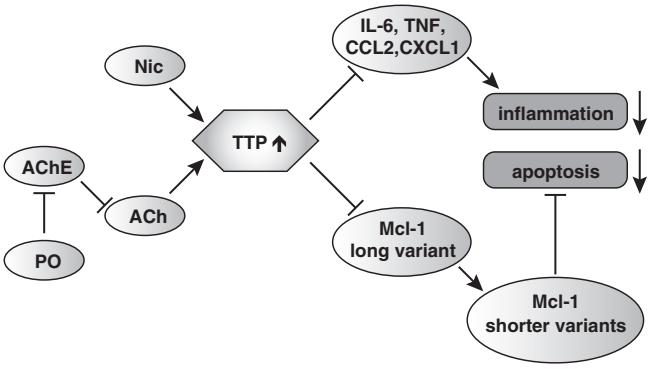

Fig. 5. TTP is a potential mediator for the cholinergic effect on apoptosis and inflammation. Cholinergic stimulation in muscles can result from increased activation of the nicotinic receptors by ACh or nicotine (Nic). Alternatively, it can result from inhibition of the ACh-hydrolyzing enzyme, AChE with Paraoxon (PO) or other inhibitors. In turn, cholinergic stimulation may reduce mRNA expression levels of cytokines and the long variant of Mcl-1 by inducing their negative regulator, TTP. This can result in reduced inflammation and apoptosis, respectively.

reduced basal CXCL1 secretion, and both basal and inflammation-induced secretion of CCL2. Thus, it is possible that impairment of nicotinic activation of muscle cells, as occurs in myasthenia gravis [64] or muscle injuries [65], chronically exposes muscle cells to high levels of chemokines while disrupting their ability to suppress inflammatory induction under secondary insults. This may explain the development of chronic inflammation and enhanced cell death in these conditions [1,13]. In agreement with that, exposure to the ACh receptor antibody in a rat model of myasthenia gravis was found to increase CCL2 expression in skeletal muscle cells [66]. In turn, CCL2 attracts T-cells and monocytes into the muscle, encouraging enhanced cytokines expression and exacerbates the pathology [67]. Indeed, increased expression of both CCL2 and CXCL1 in endothelial cells and in infiltrating immune cells within muscle tissue that is observed in several myopathies [68] may be attributed to the impaired nicotinic signaling. Additionally, CCL2 has a central role in the communication between adipose tissue and skeletal muscles [69]. CCL2 is over-expressed in obese rodents and people, and its binding to its receptor, CCR2, can trigger insulin resistance in skeletal muscles, which is part of the 'metabolic syndrome' and can precede type 2 diabetes [70]. Thus, cholinergic control over CCL2 expression in muscles further suggest that cholinergic changes might also play a prominent role in the development of insulin resistance in muscles. Indeed, serum butyrylcholinesterase levels were associated with indicators of the metabolic syndrome such as abdominal obesity and insulin concentration in the plasma [71].

Nicotinic control over IL-6 expression, as was demonstrated in nicotine-treated C2C12 cells, suggests that normal cholinergic signaling in the NMJ is important also for controlling exercise-induced effects on systemic inflammation and metabolism, which are largely mediated by muscle IL-6 secretion [12]. Moreover, it is possible that exercise-induced changes in peripheral AChE [72] are involved in the regulation of muscle IL-6 secretion during physical activity.

Our results demonstrated nicotine-mediated suppression of NF-κB and AP-1 activity, as was already shown in other cell types [73]. However, this suppression could not fully account for the suppressive effect of nicotine on pro-inflammatory transcripts, since cells with ablated TTP expression did not show this suppressive effect. Thus, TTP induction and NF-κB/AP-1 suppression may jointly act to reduce pro-inflammatory transcript levels. However, TTP interrupts NF-κB translocation and transcriptional activity [74,75], whereas NF-κB may be involved in TTP expression [76]. Therefore, nicotinic stimulation affecting one of these factors would influence the other as well.

Traditionally, TTP is regarded as a negative regulator of inflammation, as it suppresses several pro-inflammatory cytokines such as TNFα [29] and IL-6 [30], and since it negatively regulates the activity

of NF-κB [74,75]. However, its effect on inflammation appears to be more complex, as it also promotes the degradation of the anti-inflammatory cytokine IL-10 [62] and of its own transcript [77]. Moreover, TTP induction is bi-phasic; its expression is induced by the p38-MAPK pathway, but proteins of this pathway also phosphorylate TTP, holding it in a latent state. Only when the activity in this pathway is diminished, TTP is de-phosphorylated and becomes active [77,78]. Therefore, inflammatory stimuli, such as LPS, trigger the expression of pro-inflammatory genes but at the same time trigger TTP expression as a 'preparation of the antidote' for preventing chronic inflammation. In our study, TTP-deficient C2C12 cells failed to response to LPS, in striking contrast to our expectations. Thus, these findings suggest that normal basal TTP expression is essential also for the pro-inflammatory response in these cells, and raise the interesting possibility of a 'safety mechanism' ensuring that when the muscle cell is incapable of generating an anti-inflammatory response in advance (due to reduced TTP expression, and maybe in other cases as well), it limits the preceding inflammatory reaction in the first place.

Our microarray analysis revealed that the PO-induced changes in mRNA processing factors and apoptosis-related transcripts were mostly reversed or attenuated by six hours post-treatment. This is in agreement with former studies of our group, indicating that the behavioral symptoms diminish at similar time points [36]. Importantly, both AChE-R and PO injections influence the cholinergic state in the NMJ, but also have other effects; PO injection leads to AChE inhibition in the NMJ and in the muscle fibers themselves [53], resulting in higher levels of ACh and enhanced activation of muscle cholinergic receptors [4]. However, PO also exerts cytotoxic effects which are independent of the cholinergic system [79]. Additionally, changes in AChE levels, exerted by either direct AChE-R injection or by PO inhibition, have implications outside the cholinergic system, due to other cellular roles of AChE [4]. For example, lower levels of ACh in the NMJ due to AChE-R injection may disrupt muscle cell survival, but at the same time AChE-R itself is involved both in apoptotic [80] and cell proliferation [81] processes in different tissues. Similarly, higher levels of ACh in the NMJ due to PO injection are expected to promote cell survival, but at the same time the cytotoxic effects of PO induce cell death [82]. Accordingly, we detected AChE-R and PO-mediated changes in apoptosis-related transcripts, but could not classify their total effect as pro- or anti-apoptotic. Intriguingly, when AChE-R is injected together with PO, it does reverse many of PO-mediated changes. Also, we detected a significant correlation between the AChE and PO effects on mRNA processing factors and apoptosis-related transcripts, although these two treatments caused opposite cholinergic consequences. This could also be explained by the rapid feedback response induced by AChE inhibition [53,54], which encourages AChE production

Three mRNA processing factors, namely Sf3a1, SRp55 and Rnpc2, and one pro-apoptotic transcript ('Fas-long') were altered under excessive levels of AChE-R both in muscle tissues and neuronal precursor cells. Moreover, all of these transcripts were significantly changed by excessive expression of the other, more prevalent AChE variant, AChE-S, in P19 neuronal precursors [42]. Thus, these transcripts may be regulated by cholinergic stimulation independently of the physiological context. Muscarinic stimulation was shown to attenuate Fas induction in myocytes following oxidative stress [83]. Moreover, myasthenia gravis patients with anti-ACh receptor antibody have higher proportion of thymocytes expressing higher levels of Fas [84]. Further research is needed to determine whether Fas expression is globally affected by cholinergic signaling, and whether this control is mediated by Clk1 and/or its target proteins.

Our investigation of changes in apoptotic-related transcripts exerted by cholinergic-induced mRNA processing factors highlighted a previously unknown regulation mechanism for the anti-apoptotic gene, Mcl-1. Previous studies showed that Mcl-1 has a very short half-life time due to rapid degradation by the proteasome and caspases [85,86]. Here, we emphasize the importance of post-transcriptional processes such as alternative selection between different polyadenylation sites. Since 3′-UTRs are involved in mRNA stability and localization, the differences between the resulting variants have fundamental effects on Mcl-1 expression. Interestingly, recent studies suggest that cancer cells and other proliferating cells tend to express mRNA isoforms with shorter 3′-UTRs to escape from miRNA-mediated regulation [87,88]. Similarly, we identified a putative TTP binding element in the distal part of Mcl-1 3′-UTR, raising the possibility that only the long variant may be subjected to TTP-mediated degradation. Additionally, TTP expression was induced while the long variant of Mcl-1 was reduced in PO treated muscles. Since Mcl-1 is an anti-apoptotic protein, and its expression is increased in several cancer cells [89], it is possible that these cells also use the 'strategy' of switching to the short variant to escape TTP regulation. Importantly, in several tissues, Mcl-1 transcription is regulated by GM-CSF [90], which is a TTP target by itself [91]. However, TTP did not totally eliminate Mcl-1 transcripts.

5. Conclusions

Our study sheds new light on cholinergic-dependent changes in mRNA processing factors that control inflammation and apoptosis. It introduces three novel findings: (1) Cholinergic stimulation has an anti-inflammatory effect in muscle cells. (2) TTP is an essential mediator of the cholinergic anti-inflammatory pathway in muscles. (3) The Mcl-1 gene has three functional polyadenylation sites, and can be regulated by alternative selection among them under the influence of cholinergic stimulation. Together, our results emphasize the role of ACh not only as muscle activator, but also as a keeper of homeostasis in this tissue, and explain why disruption of cholinergic stimulation to muscles, as in myasthenia gravis or injuries, enhances inflammation and apoptosis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamcr.2011.11.001.

References

[1] H. Duan, J. Chai, Z. Sheng, Y. Yao, H. Yin, L. Liang, C. Shen, J. Lin, Effect of burn injury on apoptosis and expression of apoptosis-related genes/proteins in skeletal muscles of rats, Apoptosis 14 (2009) 52–65.
[2] J.L. De Bleecker, B. De Paepe, I.E. Vanwalleghem, J.M. Schroder, Differential expression of chemokines in inflammatory myopathies, Neurology 58 (2002) 1779–1785.
[3] M.M. Rich, The control of neuromuscular transmission in health and disease, Neuroscientist 12 (2006) 134–142.
[4] H. Soreq, S. Seidman, Acetylcholinesterase — new roles for an old actor, Nat. Rev. Neurosci. 2 (2001) 294–302.
[5] M. Gallowitsch-Puerta, V.A. Pavlov, Neuro-immune interactions via the cholinergic anti-inflammatory pathway, Life Sci. 80 (2007) 2325–2329.

[6] I. Shaked, A. Meerson, Y. Wolf, R. Avni, D. Greenberg, A. Gilboa-Geffen, H. Soreq, MicroRNA-132 potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase, Immunity 31 (2009) 965–973.
[7] P. Song, H.S. Sekhon, A. Lu, J. Arredondo, D. Sauer, C. Gravett, G.P. Mark, S.A. Grando, E.R. Spindel, M3 muscarinic receptor antagonists inhibit small cell lung carcinoma growth and mitogen-activated protein kinase phosphorylation induced by acetylcholine secretion, Cancer Res. 67 (2007) 3936–3944.
[8] J.J. Liu, D.L. Li, J. Zhou, L. Sun, M. Zhao, S.S. Kong, Y.H. Wang, X.J. Yu, J. Zhou, W.J. Zang, Acetylcholine prevents angiotensin II-induced oxidative stress and apoptosis in H9c2 cells, Apoptosis 16 (2010) 94–103.
[9] M. Marino, F. Scuderi, C. Provenzano, E. Bartoccioni, Skeletal muscle cells: from local inflammatory response to active immunity, Gene Ther. 18 (2010) 109–116.
[10] P. Englund, E. Lindroos, I. Nennesmo, L. Klareskog, I.E. Lundberg, Skeletal muscle fibers express major histocompatibility complex class II antigens independently of inflammatory infiltrates in inflammatory myopathies, Am. J. Pathol. 159 (2001) 1263–1273.
[11] H. Wiendl, R. Hohlfeld, B.C. Kieseier, Immunobiology of muscle: advances in understanding an immunological microenvironment, Trends Immunol. 26 (2005) 373–380.
[12] B.K. Pedersen, M.A. Febbraio, Muscle as an endocrine organ: focus on muscle-derived interleukin-6, Physiol. Rev. 88 (2008) 1379–1406.
[13] S. Acharyya, S.A. Villalta, N. Bakkar, T. Bupha-Intr, P.M. Janssen, M. Carathers, Z.W. Li, A.A. Beg, S. Ghosh, Z. Sahenk, M. Weinstein, K.L. Gardner, J.A. Rafael-Fortney, M. Karin, J.G. Tidball, A.S. Baldwin, D.C. Guttridge, Interplay of IKK/NF-kappaB signaling in macrophages and myofibers promotes muscle degeneration in Duchenne muscular dystrophy, J. Clin. Invest. 117 (2007) 889–901.
[14] K.J. Tracey, Reflex control of immunity, Nat. Rev. Immunol. 9 (2009) 418–428.
[15] P.E. Leite, J. Lagrota-Candido, L. Moraes, L. D'Elia, D.F. Pinheiro, R.F. da Silva, E.N. Yamasaki, T. Quirico-Santos, Nicotinic acetylcholine receptor activation reduces skeletal muscle inflammation of mdx mice, J. Neuroimmunol. 227 (2010) 44–51.
[16] C.O. Asomugha, D.M. Linn, C.L. Linn, ACh receptors link two signaling pathways to neuroprotection against glutamate-induced excitotoxicity in isolated RGCs, J. Neurochem. 112 (2010) 214–226.
[17] M. da Penha Berzaghi, J. Cooper, E. Castren, F. Zafra, M. Sofroniew, H. Thoenen, D. Lindholm, Cholinergic regulation of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) but not neurotrophin-3 (NT-3) mRNA levels in the developing rat hippocampus, J. Neurosci. 13 (1993) 3818–3826.
[18] M. Sandri, A.H. El Meslemani, C. Sandri, P. Schjerling, K. Vissing, J.L. Andersen, K. Rossini, U. Carraro, C. Angelini, Caspase 3 expression correlates with skeletal muscle apoptosis in Duchenne and facioscapulo human muscular dystrophy. A potential target for pharmacological treatment? J. Neuropathol. Exp. Neurol. 60 (2001) 302–312.
[19] P.M. Siu, E.W. Tam, B.T. Teng, X.M. Pei, J.W. Ng, I.F. Benzie, A.F. Mak, Muscle apoptosis is induced in pressure-induced deep tissue injury, J. Appl. Physiol. 107 (2009) 1266–1275.
[20] G.L. Warren, M. Summan, X. Gao, R. Chapman, T. Hulderman, P.P. Simeonova, Mechanisms of skeletal muscle injury and repair revealed by gene expression studies in mouse models, J. Physiol. 582 (2007) 825–841.
[21] M.J. Moore, Q. Wang, C.J. Kennedy, P.A. Silver, An alternative splicing network links cell-cycle control to apoptosis, Cell 142 (2010) 625–636.
[22] J.R. Leeman, T.D. Gilmore, Alternative splicing in the NF-kappaB signaling pathway, Gene 423 (2008) 97–107.
[23] R. Daoud, M. Da Penha Berzaghi, F. Siedler, M. Hubener, S. Stamm, Activity-dependent regulation of alternative splicing patterns in the rat brain, Eur. J. Neurosci. 11 (1999) 788–802.
[24] G. Zimmerman, M. Njunting, S. Ivens, E.A. Tolner, C.J. Behrens, M. Gross, H. Soreq, U. Heinemann, A. Friedman, Acetylcholine-induced seizure-like activity and modified cholinergic gene expression in chronically epileptic rats, Eur. J. Neurosci. 27 (2008) 965–975.
[25] D.F. Roe, G.L. Craviso, J.C. Waymire, Nicotinic stimulation modulates tyrosine hydroxylase mRNA half-life and protein binding to the 3′UTR in a manner that requires transcription, Brain Res. Mol. Brain Res. 120 (2004) 91–102.
[26] S.L. Clement, C. Scheckel, G. Stoecklin, J. Lykke-Andersen, Phosphorylation of tristetraprolin by MK2 impairs AU-rich element mRNA decay by preventing deadenylase recruitment, Mol. Cell. Biol. 31 (2011) 256–266.
[27] J. Lykke-Andersen, E. Wagner, Recruitment and activation of mRNA decay enzymes by two ARE-mediated decay activation domains in the proteins TTP and BRF-1, Genes Dev. 19 (2005) 351–361.
[28] Q. Jing, S. Huang, S. Guth, T. Zarubin, A. Motoyama, J. Chen, F. Di Padova, S.C. Lin, H. Gram, J. Han, Involvement of microRNA in AU-rich element-mediated mRNA instability, Cell 120 (2005) 623–634.
[29] E. Carballo, P.J. Blackshear, Roles of tumor necrosis factor-alpha receptor subtypes in the pathogenesis of the tristetraprolin-deficiency syndrome, Blood 98 (2001) 2389–2395.
[30] I. Sauer, B. Schaljo, C. Vogl, I. Gattermeier, T. Kolbe, M. Muller, P.J. Blackshear, P. Kovarik, Interferons limit inflammatory responses by induction of tristetraprolin, Blood 107 (2006) 4790–4797.
[31] R.L. Ogilvie, M. Abelson, H.H. Hau, I. Vlasova, P.J. Blackshear, P.R. Bohjanen, Tristetraprolin down-regulates IL-2 gene expression through AU-rich element-mediated mRNA decay, J. Immunol. 174 (2005) 953–961.
[32] N. Al-Souhibani, W. Al-Ahmadi, J.E. Hesketh, P.J. Blackshear, K.S. Khabar, The RNA-binding zinc-finger protein tristetraprolin regulates AU-rich mRNAs involved in breast cancer-related processes, Oncogene 29 (2010) 4205–4215.
[33] H.H. Lee, M.T. Vo, H.J. Kim, U.H. Lee, C.W. Kim, H.K. Kim, M.S. Ko, W.H. Lee, S.J. Cha, Y.J. Min, D.H. Choi, H.S. Suh, B.J. Lee, J.W. Park, W.J. Cho, Stability of the LATS2 tumor suppressor gene is regulated by tristetraprolin, J. Biol. Chem. 285 (2010) 17329–17337.
[34] C.W. Kim, H.K. Kim, M.T. Vo, H.H. Lee, H.J. Kim, Y.J. Min, W.J. Cho, J.W. Park, Tristetraprolin controls the stability of cIAP2 mRNA through binding to the 3′UTR of cIAP2 mRNA, Biochem. Biophys. Res. Commun. 400 (2010) 46–52.
[35] Y. Joe, H.J. Kim, S. Kim, J. Chung, M.S. Ko, W.H. Lee, K.C. Chang, J.W. Park, H.T. Chung, Tristetraprolin mediates the anti-inflammatory effects of nicotine in LPS-stimulated macrophages, J. Biol. Chem. 286 (2011) 24735–24742.
[36] T. Evron, B.C. Geyer, I. Cherni, M. Muralidharan, J. Kilbourne, S.P. Fletcher, H. Soreq, T.S. Mor, Plant-derived human acetylcholinesterase-R provides protection from lethal organophosphate poisoning and its chronic aftermath, FASEB J. 21 (2007) 2961–2969.
[37] N.L. Siow, R.C. Choi, A.W. Cheng, J.X. Jiang, D.C. Wan, S.Q. Zhu, K.W. Tsim, A cyclic AMP-dependent pathway regulates the expression of acetylcholinesterase during myogenic differentiation of C2C12 cells, J. Biol. Chem. 277 (2002) 36129–36136.
[38] E.M. King, M. Kaur, W. Gong, C.F. Rider, N.S. Holden, R. Newton, Regulation of tristetraprolin expression by interleukin-1 beta and dexamethasone in human pulmonary epithelial cells: roles for nuclear factor-kappa B and p38 mitogen-activated protein kinase, J. Pharmacol. Exp. Ther. 330 (2009) 575–585.
[39] K.R. Mahtani, M. Brook, J.L. Dean, G. Sully, J. Saklatvala, A.R. Clark, Mitogen-activated protein kinase p38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha mRNA stability, Mol. Cell. Biol. 21 (2001) 6461–6469.
[40] H. Zhang, J. Hu, M. Recce, B. Tian, PolyA_DB: a database for mammalian mRNA polyadenylation, Nucleic Acids Res. 33 (2005) D116–D120.
[41] V. Le Texier, J.J. Riethoven, V. Kumanduri, C. Gopalakrishnan, F. Lopez, D. Gautheret, T.A. Thanaraj, AltTrans: transcript pattern variants annotated for both alternative splicing and alternative polyadenylation, BMC Bioinformatics 7 (2006) 169.
[42] S. Ben-Ari, D. Toiber, A.S. Sas, H. Soreq, Y. Ben-Shaul, Modulated splicing-associated gene expression in P19 cells expressing distinct acetylcholinesterase splice variants, J. Neurochem. 97 (Suppl. 1) (2006) 24–34.
[43] D. Toiber, G. Azkona, S. Ben-Ari, N. Toran, H. Soreq, M. Dierssen, Engineering DYRK1A overdosage yields Down syndrome-characteristic cortical splicing aberrations, Neurobiol. Dis. 40 (2010) 348–359.
[44] G.A. Churchill, Fundementals of experimental design for cDNA microarrays, Nat. Genet. 32 (2002) 490–495.
[45] A.A. Dombkowski, B.J. Thibodeau, S.L. Starcevic, R.F. Novak, Gene-specific dye bias in microarray reference designs, FEBS Lett. 560 (2004) 120–124.
[46] Y. Ben-Shaul, H. Bergman, H. Soreq, Identifying subtle interrelated changes in functional gene categories using continuous measures of gene expression, Bioinformatics 21 (2005) 1129–1137.
[47] Y. Ben-Shaul, S. BenMoyal-Segal, S. Ben-Ari, H. Bergman, H. Soreq, Adaptive acetylcholinesterase splicing patterns attenuate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinsonism in mice, EJN 23 (2006) 2915–2922.
[48] N. Jelen, J. Ule, M. Zivin, Cholinergic regulation of striatal Nova mRNAs, Neuroscience 169 (2010) 619–627.
[49] J.J. Liu, D.L. Li, J. Zhou, L. Sun, M. Zhao, S.S. Kong, Y.H. Wang, X.J. Yu, J. Zhou, W.J. Zang, Acetylcholine prevents angiotensin II-induced oxidative stress and apoptosis in H9c2 cells, Apoptosis 16 (2011) 94–103.
[50] J.M. Waldburger, D.L. Boyle, V.A. Pavlov, K.J. Tracey, G.S. Firestein, Acetylcholine regulation of synoviocyte cytokine expression by the alpha7 nicotinic receptor, Arthritis Rheum. 58 (2008) 3439–3449.
[51] C. Sadis, G. Teske, G. Stokman, C. Kubjak, N. Claessen, F. Moore, P. Loi, B. Diallo, L. Barvais, M. Goldman, S. Florquin, A. Le Moine, Nicotine protects kidney from renal ischemia/reperfusion injury through the cholinergic anti-inflammatory pathway, PLoS One 2 (2007) e469.
[52] S. Datta, R. Biswas, M. Novotny, P.G. Pavicic Jr., T. Herjan, P. Mandal, T.A. Hamilton, Tristetraprolin regulates CXCL1 (KC) mRNA stability, J. Immunol. 180 (2008) 2545–2552.
[53] C.M. Cisson, B.W. Wilson, Paraoxon increases the rate of synthesis of acetylcholinesterase in cultured muscle, Toxicol. Lett. 9 (1981) 131–135.
[54] D. Kaufer, A. Friedman, S. Seidman, H. Soreq, Anticholinesterases induce multigenic transcriptional feedback response suppressing cholinergic neurotransmission, Chem. Biol. Interact. 119–120 (1999) 349–360.
[55] D. Lleres, M. Denegri, M. Biggiogera, P. Ajuh, A.I. Lamond, Direct interaction between hnRNP-M and CDC5L/PLRG1 proteins affects alternative splice site choice, EMBO Rep. 11 (2010) 445–451.
[56] A. Kleinridders, H.M. Pogoda, S. Irlenbusch, N. Smyth, C. Koncz, M. Hammerschmidt, J.C. Bruning, PLRG1 is an essential regulator of cell proliferation and apoptosis during vertebrate development and tissue homeostasis, Mol. Cell. Biol. 29 (2009) 3173–3185.
[57] S. Le Gouill, K. Podar, J.L. Harousseau, K.C. Anderson, Mcl-1 regulation and its role in multiple myeloma, Cell Cycle 3 (2004) 1259–1262.
[58] S. Kojima, A. Hyakutake, N. Koshikawa, A. Nakagawara, K. Takenaga, MCL-1V, a novel mouse antiapoptotic MCL-1 variant, generated by RNA splicing at a non-canonical splicing pair, Biochem. Biophys. Res. Commun. 391 (2010) 492–497.
[59] A. De Biasio, J.A. Vrana, P. Zhou, L. Qian, C.K. Bieszczad, K.E. Braley, A.M. Domina, S.J. Weintraub, J.M. Neveu, W.S. Lane, R.W. Craig, N-terminal truncation of antiapoptotic MCL1, but not G2/M-induced phosphorylation, is associated with stabilization and abundant expression in tumor cells, J. Biol. Chem. 282 (2007) 23919–23936.
[60] B.Y. Brewer, J.D. Ballin, E.J. Fialcowitz-White, P.J. Blackshear, G.M. Wilson, Substrate dependence of conformational changes in the RNA-binding domain of tristetraprolin assessed by fluorescence spectroscopy of tryptophan mutants, Biochemistry 45 (2006) 13807–13817.
[61] J. Emmons, W.H. Townley-Tilson, K.M. Deleault, S.J. Skinner, R.H. Gross, M.L. Whitfield, S.A. Brooks, Identification of TTP mRNA targets in human dendritic cells reveals TTP as a critical regulator of dendritic cell maturation, RNA 14 (2008) 888–902.

[62] G. Stoecklin, S.A. Tenenbaum, T. Mayo, S.V. Chittur, A.D. George, T.E. Baroni, P.J. Blackshear, P. Anderson, Genome-wide analysis identifies interleukin-10 mRNA as target of tristetraprolin, J. Biol. Chem. 283 (2008) 11689–11699.
[63] H. Tsuneki, R. Salas, J.A. Dani, Mouse muscle denervation increases expression of an alpha7 nicotinic receptor with unusual pharmacology, J. Physiol. 547 (2003) 169–179.
[64] J.M. Lindstrom, Acetylcholine receptors and myasthenia, Muscle Nerve 23 (2000) 453–477.
[65] J.M. Ward, J.A. Martyn, Burn injury-induced nicotinic acetylcholine receptor changes on muscle membrane, Muscle Nerve 16 (1993) 348–354.
[66] S. Reyes-Reyna, T. Stegall, K.A. Krolick, Muscle responds to an antibody reactive with the acetylcholine receptor by up-regulating monocyte chemoattractant protein 1: a chemokine with the potential to influence the severity and course of experimental myasthenia gravis, J. Immunol. 169 (2002) 1579–1586.
[67] S. Shandley, S. Martinez, K. Krolick, IL-4 receptor as a bridge between the immune system and muscle in experimental myasthenia gravis I: up-regulation of muscle IL-15 by IL-4, Clin. Immunol. 132 (2009) 246–256.
[68] B. De Paepe, K.K. Creus, J.L. De Bleecker, Chemokine profile of different inflammatory myopathies reflects humoral versus cytotoxic immune responses, Ann. N. Y. Acad. Sci. 1109 (2007) 441–453.
[69] H. Sell, D. Dietze-Schroeder, U. Kaiser, J. Eckel, Monocyte chemotactic protein-1 is a potential player in the negative cross-talk between adipose tissue and skeletal muscle, Endocrinology 147 (2006) 2458–2467.
[70] H. Sell, J. Eckel, Monocyte chemotactic protein-1 and its role in insulin resistance, Curr. Opin. Lipidol. 18 (2007) 258–262.
[71] A. Valle, D.T. O'Connor, P. Taylor, G. Zhu, G.W. Montgomery, P.E. Slagboom, N.G. Martin, J.B. Whitfield, Butyrylcholinesterase: association with the metabolic syndrome and identification of 2 gene loci affecting activity, Clin. Chem. 52 (2006) 1014–1020.
[72] S. Ben-Ari, K. Ofek, S. Barbash, H. Meiri, E. Kovalev, D.S. Greenberg, H. Soreq, S. Shoham, Similar cation channels mediate protection from cerebellar exitotoxicity by exercise and inheritance, J. Cell. Mol. Med. (2011), doi:10.1111/j.1582-4934.2011.01331.x.
[73] R. Ravikumar, G. Flora, J.W. Geddes, B. Hennig, M. Toborek, Nicotine attenuates oxidative stress, activation of redox-regulated transcription factors and induction of proinflammatory genes in compressive spinal cord trauma, Brain Res. Mol. Brain Res. 124 (2004) 188–198.
[74] J. Liang, T. Lei, Y. Song, N. Yanes, Y. Qi, M. Fu, RNA-destabilizing factor tristetraprolin negatively regulates NF-kappaB signaling, J. Biol. Chem. 284 (2009) 29383–29390.
[75] Y.M. Schichl, U. Resch, R. Hofer-Warbinek, R. de Martin, Tristetraprolin impairs NF-kappaB/p65 nuclear translocation, J. Biol. Chem. 284 (2009) 29571–29581.
[76] W.S. Lai, M.J. Thompson, P.J. Blackshear, Characteristics of the intron involvement in the mitogen-induced expression of Zfp-36, J. Biol. Chem. 273 (1998) 506–517.
[77] M. Brook, C.R. Tchen, T. Santalucia, J. McIlrath, J.S. Arthur, J. Saklatvala, A.R. Clark, Posttranslational regulation of tristetraprolin subcellular localization and protein stability by p38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways, Mol. Cell. Biol. 26 (2006) 2408–2418.
[78] F.P. Marchese, A. Aubareda, C. Tudor, J. Saklatvala, A.R. Clark, J.L. Dean, MAPKAP kinase 2 blocks tristetraprolin-directed mRNA decay by inhibiting CAF1 deadenylase recruitment, J. Biol. Chem. 285 (2010) 27590–27600.
[79] U. Undeger, N. Basaran, Effects of pesticides on human peripheral lymphocytes in vitro: induction of DNA damage, Arch. Toxicol. 79 (2005) 169–176.
[80] K. Pegan, U. Matkovic, T. Mars, K. Mis, S. Pirkmajer, J. Brecelj, Z. Grubic, Acetylcholinesterase is involved in apoptosis in the precursors of human muscle regeneration, Chem. Biol. Interact. 187 (2010) 96–100.
[81] D. Grisaru, V. Deutsch, M. Shapira, M. Pick, M. Sternfeld, N. Melamed-Book, D. Kaufer, N. Galyam, M.J. Gait, D. Owen, J.B. Lessing, A. Eldor, H. Soreq, ARP, a peptide derived from the stress-associated acetylcholinesterase variant, has hematopoietic growth promoting activities, Mol. Med. 7 (2001) 93–105.
[82] A.M. Saleh, C. Vijayasarathy, L. Masoud, L. Kumar, A. Shahin, A. Kambal, Paraoxon induces apoptosis in EL4 cells via activation of mitochondrial pathways, Toxicol. Appl. Pharmacol. 190 (2003) 47–57.
[83] B. Yang, H. Lin, C. Xu, Y. Liu, H. Wang, H. Han, Z. Wang, Choline produces cytoprotective effects against ischemic myocardial injuries: evidence for the role of cardiac m3 subtype muscarinic acetylcholine receptors, Cell. Physiol. Biochem. 16 (2005) 163–174.
[84] N. Moulian, J. Bidault, F. Truffault, A.M. Yamamoto, P. Levasseur, S. Berrih-Aknin, Thymocyte Fas expression is dysregulated in myasthenia gravis patients with anti-acetylcholine receptor antibody, Blood 89 (1997) 3287–3295.
[85] S.N. Willis, L. Chen, G. Dewson, A. Wei, E. Naik, J.I. Fletcher, J.M. Adams, D.C. Huang, Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins, Genes Dev. 19 (2005) 1294–1305.
[86] M. Rahmani, E.M. Davis, C. Bauer, P. Dent, S. Grant, Apoptosis induced by the kinase inhibitor BAY 43-9006 in human leukemia cells involves down-regulation of Mcl-1 through inhibition of translation, J. Biol. Chem. 280 (2005) 35217–35227.
[87] R. Sandberg, J.R. Neilson, A. Sarma, P.A. Sharp, C.B. Burge, Proliferating cells express mRNAs with shortened 3′ untranslated regions and fewer microRNA target sites, Science 320 (2008) 1643–1647.
[88] S. Goswami, R.S. Tarapore, J.J. Teslaa, Y. Grinblat, V. Setaluri, V.S. Spiegelman, MicroRNA-340-mediated degradation of microphthalmia-associated transcription factor mRNA is inhibited by the coding region determinant-binding protein, J. Biol. Chem. 285 (2010) 20532–20540.
[89] M.R. Warr, G.C. Shore, Unique biology of Mcl-1: therapeutic opportunities in cancer, Curr. Mol. Med. 8 (2008) 138–147.
[90] C. Akgul, P.C. Turner, M.R. White, S.W. Edwards, Functional analysis of the human MCL-1 gene, Cell. Mol. Life Sci. 57 (2000) 684–691.
[91] E. Carballo, W.S. Lai, P.J. Blackshear, Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability, Blood 95 (2000) 1891–1899.