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# Human muscle macrophages express β-amyloid precursor and prion proteins and their mRNAs

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WELL-CHARACTERIZED antibodies against β-amyloid precursor protein (βAPP) and prion protein (PrP), and specific cRNA probes, were used to localize βAPP and PrP and their mRNAs in human muscle macrophages. Macrophages present in muscle biopsies of 51 patients with various neuromuscular disorders showed accumulation of βAPP and PrP, and strongly expressed βAPP and PrP mRNAs. These were present in all muscle macrophages unrelated to their localization within the muscle tissue or diagnosis. Our study provides the first demonstration that human muscle resident macrophages synthesize and accumulate βAPP and PrP. We suggest that those proteins play a role in biology of muscle macrophages, including their participation in inflammatory and immune responses.

**Key words:** Macrophages; Human muscle; βAPP; βAPP mRNA; Prion protein; Prion protein mRNA



## Introduction

Tissue macrophages have several important functions. In addition to their key role in humoral and cell-mediated immune responses, they possess strong microbiocidal function and play an essential role in phagocytosis of damaged cells (reviewed in Ref. 1). Macrophages are tissue-specific and derived from circulating monocytes. Upon entry of monocytes into the extravascular space of a given tissue, they are transformed into resident macrophages and acquire some tissue-specific characteristics depending on the milieu in which they reside.[1,2] Discovering the tissue characteristics of macrophages may be helpful in understanding their role in the pathogenesis of various tissue-specific inflammatory and immune-mediated diseases.

Muscle macrophages contribute to the inflammatory responses in polymyositis, dermatomyositis, inclusion-body myositis, sarcoid myositis and other immune-mediated myopathies (reviewed in Ref. 3), but their biological characteristics are not well known. We now describe new qualitative characteristics of human muscle macrophages, namely their *in vivo* synthesis and accumulation of β-amyloid precursor protein (βAPP) and prion protein (PrP), suggesting that these proteins are important in their biological functions.

## Materials and Methods

*Patients*: We studied diagnostic muscle biopsies of 32 males and 19 females, ages 5–79 years, with the following diagnoses: polymyositis (10), dermatomyositis (2), sporadic inclusion-body myositis (10), hereditary inclusion-body myopathy (5), Duchenne muscular dystrophy (2), morphologically non-specific myopathy (5), amyotrophic lateral sclerosis (9) and normal (8). Diagnosis was based on clinical, laboratory, muscle biopsy 18-reaction histochemistry, electron microscopy and immunocytochemical studies.

*In situ hybridization*: *In situ* hybridization was performed as described previously,[4,5] on 10 μm transverse sections of fresh-frozen muscle biopsies. In brief, sections were fixed in 4% paraformaldehyde, treated with 0.5% acidic anhydride in 0.1 mol $l^{-1}$ triethanolamine (pH 8.0), and prehybridized for 1 h at 55°C. Hybridization was performed for 3 h in prehybridization buffer (50% formamide, 0.75 mol $l^{-1}$ NaCl, 0.05 mol $l^{-1}$ sodium phosphate buffer pH 7.0, 10 mmol $l^{-1}$ EDTA, 200 μg $ml^{-1}$ poly-A, 25 μg ml poly-C, and 500 μg $ml^{-1}$ purified tRNA) containing 10% dextran sulfate and [$^{35}$S]RNA probe ($2 \times 10^6$ c.p.m. $ml^{-1}$). After hybridization, sections were treated with 20 μg $ml^{-1}$ RNase A, washed under high stringency conditions at 60°C for 1 h, and agitated overnight in 1 × SSC. The dehydrated sections were coated with NTB-2 emulsion and exposed for 8–10 weeks.

*βAPP and PrP ribonucleotide probes*: Three previously described[4] human βAPP RNA probes were used in this study. Probe J-24 hybridizes to the βAPP-695 mRNA, probe I-22 hybridizes to βAPP mRNA encoding the Kunitz protease inhibitor (KPI) domain, and probe 'βAPP-751' hybridizes to all differentially spliced βAPP mRNAs. cRNA probes were transcribed from cDNA clones using SP6 and T7RNA polymerases (Bethesda Research Laboratories, Gaithesburg, MD). The I-22 and J-24 pGEM-I recombinant vectors were constructed by ligation of double-stranded oligonu-

cleotides containing pre-formed *Eco*RI and *Hind*III sites. I-22 contained βAPP 751 nt 1062–1092 and J-24 contained βAPP 751 nt 975–989 plus nt 1158–1172 sequences.[4] The third βAPP cDNA clone contained the 2.9 kb *Sma*I–*Hind*III cDNA fragment of the full-length βAPP-751 sequence inserted into pGEM-3 plasmid, and hybridized to all differentially-spliced βAPP mRNAs. For all three probes, both antisense and sense RNA probes were made as described[4] and used throughout.

The PrP ribroprobe has also been described previously.[5] In brief, we isolated a full-length, human PrPcDNA sequence from pUC8 recombinant clone (HuPrPDNA2)[6] using *Eco*RI digestion. The isolated 2.4 kb DNA fragment (nt 1–2432) was inserted into pGEM-3Z (Promega) downstream to the SP6 promoter using T4 DNA ligase (Gibco BRL). Both antisense and sense RNA probes were transcribed as described[5] and were used throughout.

*Immunocytochemistry*: Light microscopy immunocytochemical staining utilizing the peroxidase–anti-peroxidase method was performed on sections parallel to those on which *in situ* hybridization was performed, following the same general procedures as described.[4,5] In addition, double-label immunofluorescence utilizing FITC and Texas red was used to co-localize two different antigens on the same muscle section.[7–9] Macrophages were identified with the monoclonal antibody Ber-MAC 3 (Dako), which recognizes a 140 kDa protein present on the surface and in the cytoplasm of tissue macrophages. To immunolocalize βAPP epitopes, including β-amyloid protein (Aβ), we used the following three rabbit-polyclonal antisera: (a) C8, directed against a synthetic peptide of C-terminal amino acids 676–695,[10] diluted 1:1000; (b) SP18, against the N-terminal sequence 45–62 of βAPP,[11] diluted 1:500 and (c) R1280, against sequence 1–40 of synthetic Aβ,[12] diluted 1:1000. PrP was immunolocalized with two antisera: (a) polyclonal antiserum R073 against scrapie PrP 27–30[13] and (b) mouse monoclonal antibody against human PrP 27–30.[14] PrP antibodies were used in dilution 1:200–1:500. All antibodies were shown to be highly specific in our previous studies.[7,9,15,16] To block non-specific binding of antibody to Fc receptors, primary antibodies were preincubated with 1:10 diluted normal goat serum before use.

Electronmicroscopy (EM) immunocytochemistry was performed on 10 μm unfixed frozen sections attached to the bottom of 35 mm Petri dishes, as described previously.[7–9] In brief, after blocking the Fc receptors with 1:10 diluted normal goat serum, the sections were incubated overnight with a mixture of either polyclonal antiserum R1280 against Aβ plus mouse monoclonal antibody Ber-MAC 3, or with a mixture of polyclonal antiserum against PrP plus Ber-MAC 3. Subsequently, sections were incubated with goat polyclonal antiserum against rabbit IgG labeled with 10 nm gold particles, followed by incubation with horseradish peroxidase (HRP) labeled goat antiserum against mouse IgG. Sections were then fixed in 2% paraformaldehyde 1.2% glutaraldehyde mixture, reacted with DAB-$H_2O_2$ to visualize HRP, postfixed in osmium, and embedded *in situ* in the Petri dish, as described.[7–9] After embedding, the section was viewed under the bright-field microscopy, the macrophages darkly stained with the HRP–DAB reaction product were identified, and the areas containing them were marked. The epon disk was removed from the dish and the marked areas were cored-drilled.[7,8] The drilled-out cores, each containing clusters of macrophages, were mounted in an epon blank block, trimmed, thin-sectioned and examined by EM without counterstain.

## Results

In all muscle biopsies, macrophages, identified by a positive staining with Ber-MAC 3 showed strong βAPP and PrP immunoreactivities with all antibodies studied, and strong βAPP and PrP mRNA signals (Fig. 1). Both the total-βAPP probe, which contains the KPI motif of βAPP, and the KPI-specific probe produced the same results. However, the J-24 probe, which hybridizes to βAPP-695 mRNA, did not show a positive signal above the weak sense-strand background. As in our previous studies, the sense-strand controls produced only uniformly weak background staining. Omitting or replacing the primary antibody produced negative results.

βAPP and PrP immunoreactivities and their mRNAs were expressed in all muscle macrophages independent of their localization within the tissue. They were present in macrophages invading normal-appearing muscle fibers, in the perivascular mononuclear inflammatory cell collections, and in single macrophages sparsely distributed around normal muscle fibers. In each muscle biopsy, and within each group of diseases, the intensity of βAPP and PrP immunoreactivities and the intensity of their mRNAs differed slightly among macrophages, perhaps reflecting different stages of their activation. However, the intensity of βAPP and PrP immunoreactivities and the intensity of their mRNA signal did not seem dependent either on the muscle disease studied, or the age or gender of the patient. The number of macrophages in a given biopsy appeared to depend on disease activity and was irregular, thus being subject to biopsy sampling.

As we have previously described,[4,5,7–9] increases in PrP and βAPP immunoreactivities and their mRNAs were also present in approximately 85% of vacuolated muscle fibers of sporadic inclusion-body myositis and hereditary inclusion-body myopathy. Also, as previously reported,[17] regenerating muscle fibers in various muscle diseases had increased βAPP and PrP immunoreactivities and increased mRNA signals.

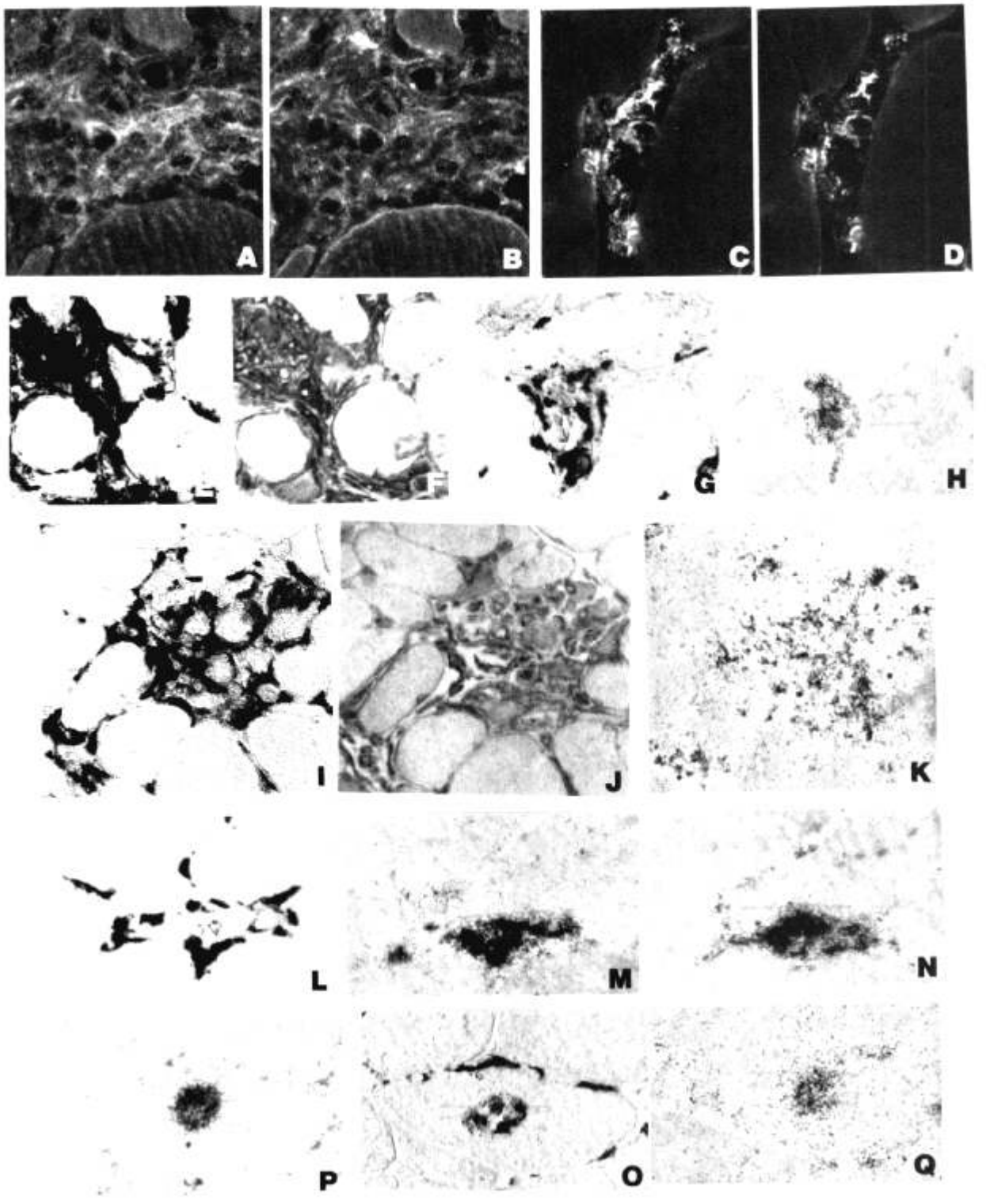


FIG. 1. Light microscopy (**A–D**) Double-labeling of macrophages with the specific marker Ber-MAC 3 (A, C), with C-terminal βAPP (B), and N-terminal βAPP (D). (**E, G, I, L, O**) Immunocytochemistry of Ber-MAC 3. (**F, J**) Immunocytochemistry of PrP; H, M, Q—*in situ* hybridization of βAPP mRNA. (**K, N, P**) *In situ* hybridization of PrP mRNA. In each group, reactions were performed on parallel (but not always adjacent) sections. Macrophages identified by Ber-MAC 3 contain strong βAPP and PrP immunoreactivity, as well as βAPP and PrP mRNA signals. A–D, ×1600; E–K, ×800; L–Q, ×1300. A–N, interstitial or perivascular mononuclear-cell inflammatory-cell collections; O–Q, a muscle fiber undergoing central phagocytosis.

By immunoelectronmicroscopy, macrophages identified by dark immunoperoxidase staining with Ber-MAC 3 contained βAPP and PrP immunoreactive material decorated with gold particles (Fig. 2). Gold particles were most commonly found either in clusters, or decorating various sized membranous and fibrillar structures. βAPP and PrP immunolocalization was confined to macrophages; the neighboring structures including collagen fibrils unstained with macrophage marker, had no βAPP and PrP immunoreactivities. The procedure used by us for immunoelectronmicroscopy enabled easy identification of macrophages due to their dark staining with a macrophage-specific marker and their double labeling with antibodies against βAPP and PrP. However, it did not permit excellent ultrastructural preservation of the macrophages because the tissue was not prefixed before immunolabeling. We have chosen this technique because ultrastructural identification of macrophages was essential for this study: even slight prefixation resulted in no immunostaining of the macrophages, presumably because it impaired their antigenic sites.

## Discussion and Conclusions

βAPP, a product of a chromosome 21 gene, is a glycoprotein cell surface component containing a large extracellular N-terminal domain, a transmembrane domain and a short cytoplasmic C-terminal domain (reviewed in Ref. 18). The βAPP gene produces at least three alternatively spliced transcripts encoding βAPP containing 695, 751 or 770 amino acids; the last two contain in their extracellular region an insert of a Kunitz-type protease inhibitor (KPI). βAPP-695 mRNA, which lacks KPI, is the predominant βAPP mRNA in brain, whereas mRNAs for βAPP classes containing KPI predominate in peripheral tissues.[18] Aβ is a 39–43 amino acid polypeptide that is a part of βAPP in its transmembrane domain.[18] The biological functions of βAPP are not yet well understood, but it may play a role in maintaining cell integrity and shape, and in cell–cell interactions.[18] The previous demonstration that βAPP epitopes and βAPP mRNA are strongly accumulated at the postsynaptic domain of human neuromuscular junctions[4,15] suggests a role in muscle–

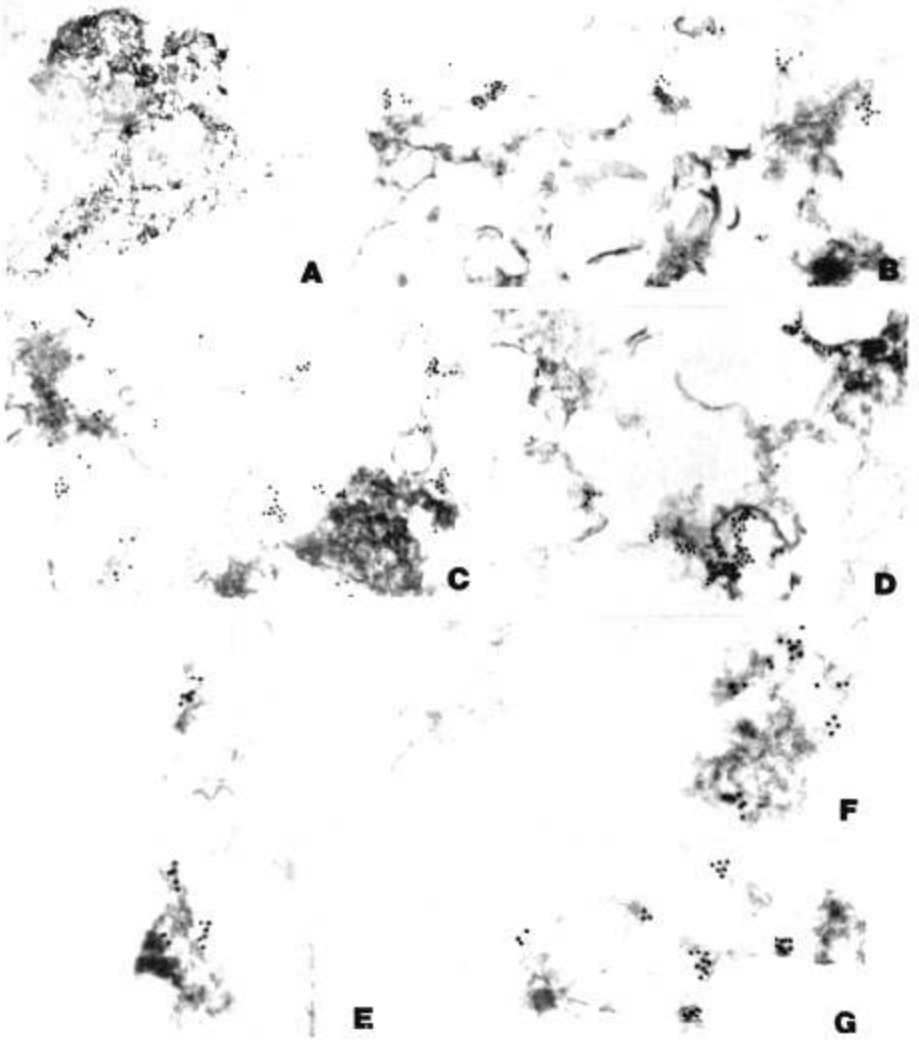


FIG. 2. Ultrastructural immunocytochemistry. (**A**) Low-power electronmicrograph illustrating macrophages darkly-stained with Ber-MAC 3 in the HRP-DAB reaction (×2580). (**B–D**) Gold immuno-EM illustrating that localization of PrP is confined to membranous material of darkly stained macrophages (×28 380). (**E–G**) Gold immuno-EM illustrating localization of Aβ on darkly-stained macrophages (E, G, ×35 260; F, ×45 580; 10 nm gold particles in all). Collagen fibrils (E, F, G) and other structures unstained with the Ber-MAC 3 marker are not decorated with immuno-gold.

nerve contact or dynamic interaction. Aβ and βAPP have been receiving considerable attention regarding the pathogenesis of Alzheimer disease. Aβ is a major component of amyloid fibrils in blood vessels and senile plaques in the brain of patients with Alzheimer disease, Down syndrome, Dutch hereditary cerebrovascular amyloidosis and very advanced age.[18]

Normal cellular prion protein ($PrP^c$) is expressed mainly in neurons. It is a 33–37 kDa membrane protein anchored by a glycophosphoinositol (reviewed in Ref. 19). Human $PrP^c$ is encoded by a single gene consisting of two exons and one intron located on the short arm of chromosome 20.[19] Its function is uncertain. In normal human muscle, strong PrP immunoreactivity and mRNA concentration at the post-synaptic domain of the neuromuscular junctions[5,16] suggests that $PrP^c$ plays a physiological role there.

PrP scrapie ($PrP^{sc}$) is a 33–37 kDa proteinase-K-resistant protein resulting from postranslational modification of $PrP^c$.[19] $PrP^{sc}$ and $PrP^c$ are encoded by the same gene,[19] and antibodies *per se* cannot distinguish between them. Postranslational modification of $PrP^c$ can be stimulated by a transmissible agent or an inherited mutation within the $PrP^c$ gene.[19] $PrP^{sc}$ is found in brains of scrapie-infected animals and patients with kuru, Creutzfeldt–Jakob disease and Gerstmann–Straussler–Scheinker syndrome.[19]

Previously, both βAPP and PrP were considered to play a role only in brain diseases. Recently, however, both βAPP and PrP and their mRNAs have been shown to be accumulated at normal human neuromuscular junctions and abnormally accumulated in inclusion-body myositis and hereditary inclusion-body myopathy.[4,5,7–9] Therefore, those proteins seem to play a role outside the central nervous system, namely in human muscle.

Our report is the first to describe the accumulation of PrP and three epitopes of βAPP, including Aβ, in human muscle macrophages. Moreover, muscle macrophages also strongly express 751-βAPP mRNA, which contains the Kunitz-type protease inhibitor insert, and $PrP^c$ mRNA, indicating that they synthesize both βAPP and $PrP^c$. 695-βAPP mRNA, predominant in the brain, is not transcribed in muscle macrophages.

Brain microglia are considered equivalent in many respects to peripheral tissue macrophages, even though several differences exist between them.[20] An immune-mediated mechanism of Alzheimer disease involving microglia has been proposed by several investigators. Aβ has been demonstrated in microglia of Alzheimer brain,[21,22] however, βAPP mRNA[23] could not be demonstrated. To our knowledge, expression of $PrP^c$ and its mRNA in brain microglia have not been described. Human circulating monocytes cultured *in vitro* were shown to synthesize βAPP.[24] Whether βAPP and PrP mRNAs are transcribed in resident macrophages of other human tissues remains to be determined.

The fact that human muscle macrophages synthesize and accumulate both βAPP and PrP suggest that those proteins play a role in the biology of muscle macrophages, including those involved in human muscle immune/inflammatory responses. Treatment of patients with the glucocorticoid prednisone reduces the inflammatory reaction in muscle diseases by various mechanisms. It is of interest that treatment of cultured human muscle with glucorticoids significantly reduces the amount of mRNAs of both βAPP[25] and $PrP^c$ (Sarkozi, McFerrin, Askanas, unpublished observations). Therefore, it is possible that glucocorticoids may also reduce synthesis of βAPP and PrP in muscle macrophages *in vivo*, which in turn may influence their behavior in immune/inflammatory conditions. Future studies regarding the influence of various factors, including glucocorticoids, on the synthesis of βAPP and PrP in muscle macrophages, both *in vitro* and *in vivo*, should be of interest.

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